ENDOCRINOLOGY

VOLUME 40 MARCH, 1947 NUMBER 3

PIGMENTATION AND HAIR GROWTH IN BLACK RATS, AS MODIFIED BY THE CHRONIC ADMINISTRATION OF THIOUREA, PHENYL THIOUREA AND ALPHA-NAPHTHYL THIOUREA[1]

SALLY H. DIEKE

Psychobiological Laboratory, Phipps Psychiatric Clinic, The Johns Hopkins Hospital

BALTIMORE, MARYLAND

OBSERVATIONS have often been made of the fact that hair growth is intermittent, so that at any particular time some hairs are growing actively while other neighboring ones are not lengthening at all. In measuring the growth of marked human hairs Trotter (1924) found that the underlying cause of this apparent irregularity is a rhythmic alternation between activity and quiescence in individual hair follicles.

In man the distribution of active and inactive follicles would seem to be random, so that of two adjacent hairs one can be growing while the other has reached its maximum length and protrudes from a temporarily inactive follicle. In many laboratory animals, however, there is a certain regional coordination in activity, with the result that at any given time hair is being produced from most if not all follicles in some areas while no growth occurs from those elsewhere. This fact was known, for instance, to Forster (1929) who used cats rather than mice, rats or rabbits to study hair growth stimulants, because the last named animals all had "irregular, islandlike" hair regrowth patterns.

The phenomena of hair growth in rodents have been most extensively studied by Dry, first in the mouse (Dry, 1926) and later confirmed in the rat (Dry, 1928–29). He observed three definite phases in follicle activity, which he called anagen, catagen and telogen. These correspond, respectively, to the period of active growth, the period of

Received for publication August 23, 1946.

[1] Started under a contract recommended by the Committee on Medical Research between the Office of Scientific Research and Development and The Johns Hopkins University and continued under a contract between the Medical Division, Chemical Corps, U. S. Army, and The Johns Hopkins University.

Copyright, 1947, by the Association for the Study of Internal Secretions

reduction in size of the hair root, and the resting period, during which the medulla-free base terminates in a bulbous enlargement called a "club." In every part of the animal's skin these three phases follow in succession, repeating again and again throughout life. An active growth phase (herein designated G_1, G_2, etc., the subscript indicating order of appearance) is found only on a limited area of the skin at any time but moves in a wavelike fashion to other areas until every follicle has become active in turn. These waves of growth begin near the midventer and proceed longitudinally up both sides to meet at the spine in the thoracic region. This leaves two islands, one around the crown of the head and the other around the sacrum, which are subsequently covered as the wave rolls in to engulf them.

These findings have been confirmed by Butcher and Richards (1939) and also by Mushett and Unna (1941). More recently Haddow et al. (1945) have demonstrated them by administering a dye which is selectively deposited in growing hair shafts.

As each successive wave passed over any given follicle, Dry noted that another hair was usually added without displacing the hairs of preceding generations. (Follicles in rats and mice are multiple; see Fraser (1931) and Butcher (1934–35).) He also found that the time necessary for a wave to cover the entire body depended upon the age and, to some extent, on the health of the animal: In general G_1 was completed more rapidly than G_2, with increasingly greater delay accompanying the completion of succeeding waves, particularly in sick individuals.

From the foregoing it is clear that the normal nonuniformity of hair production in the rat introduces complications when this animal is used to study experimental hair changes. Artificial greying, in particular, is obscured by the fact that newly growing hairs do not push out the older ones of preceding generations.

The use of solid color black or brown rats rather than albinos simplifies the study of hair growth in one respect, since in them the normal precursor of hair growth is a darkening of those parts of the skin where hair will grow (Haydak, 1935; György et al., 1940; Mushett and Unna, 1941). This darkening, which makes the various phases of follicle activity recognizable at a glance, is caused by a massing of melanin around the hair roots.[2]

The present work is a study of how normal hair and pigment cycles in black rats are influenced by the chronic administration of thiourea, phenyl thiourea and alpha-naphthyl thiourea. Considerable material on cycles in normal, untreated black rats is included for comparison, as well as some confirmatory observations made on wild Norway rats

[2] According to Steiner-Wourlisch (1925) and Strong (1927), this melanin is all in the basal cells, and not in the corium as is the pigment of Mongolian spots and of the blue patches seen on apes.

and black mice. This report covers only grossly visible effects on hair and skin; no microscopic findings are included.

METHODS

The black (non-agouti) rats used were from the colony maintained in this laboratory since 1927, which stems partly from Wistar albinos and partly from pigmented rats of Dr. E. V. McCollum's strain. They were not homozygous blacks, having a mixed black, tan and hooded ancestry.

At ages between 21 and 30 days the rats were weaned and placed in individual cages. (A few adult rats were used in initial studies.) They ate the stock diet customarily fed in this laboratory,[3] supplemented once weekly with lettuce, and drank either tap water or a distilled water solution of one of the drugs. Records were made daily of food and water intakes, and weekly of body weights. Both male and female rats were used, from litters born at all seasons of the year. Most of the normals were littermates to the chronically poisoned animals.

Observations on skin color were made by removing all the fur from the left side of each rat's body at frequent intervals. Electric clippers[4] proved to be ideal for this purpose and were, therefore, used exclusively. Each rat was examined at least once a week and the fur was clipped off whenever it had grown sufficiently to obscure the skin, which amounted to clipping younger rats every three or four days and older ones as infrequently as once a month. The rats submitted readily to clipping and required no anesthesia or any other restraint. The fur was never disturbed on the right side of the body; because of the symmetric nature of hair growth noted above, an effective control area thus existed on the body of each rat.

The removal of fur presumably did not have any inherent stimulating effect on fur growth. (Trotter (1928) found shaving did not affect the growth rate of hair) but it may have produced a difference in skin temperature between the two sides of the body and thereby led to different replacement rates on the clipped and unclipped sides. The dependence of hair growth on temperature does not seem to have been studied extensively; Eaton and Eaton (1937) found, however, that human beard hairs grow faster in summer than in winter. The present observations, made over a period of two years, have given no indications of seasonal variation. The temperature of the room where the rats were kept remained relatively constant, averaging about 78° F. and varying from an occasional low of 73° in the winter to a high of 87° on one summer day, with no sharp diurnal fluctuations.

From time to time color photographs were made of the rats (under light ether anesthesia), so that there would be permanent records of the progressive changes.

Administration of the drugs was started with small amounts, and gradually increased as tolerance became established. It was found that fewer rats died from acute poisoning when the treatment started before three months of age, because young rats have a naturally higher resistance to acute poisoning by thiourea and its derivatives than do adults. (For a discussion

[3] Graham flour 72.5%, skim milk powder 10.0%, casein 10.0%, calcium carbonate 1.5%, sodium chloride 1.0%, and butter 5.0%.

[4] Oster animal clippers, Model No. 2, size 40.

of this and for other observations on the toxic effects of these compounds see Richter and Clisby (1942), Dieke and Richter (1945), Richter (1945), and Dieke and Richter (1946).) Administration in food at concentrations as low as 0.1 per cent produced undesirable toxic effects with lowered food intakes and increasing debility. Better results were obtained when the drugs were given dissolved in the drinking water. (Alpha-naphthyl thiourea, being very insoluble, was given suspended in 10 per cent acacia solution as a daily supplement by stomach tube.) The general condition of the rats was watched carefully and the dose adjusted to minimize toxic reactions, thereby avoiding as far as possible the depressive effects on the hair which accompany marked ill health.

RESULTS I—NORMAL RATS

The cycles of twelve normal black rats were followed from birth throughout life (some are still alive). Thirty suckling black rats were also observed daily from birth to 30 days of age, and single observations, or observations covering only one or two cycles, were made on more than a hundred other rats, including wild norways. Insofar as they overlap, the findings substantiate those of Butcher (1934–35), who worked exclusively with albinos, and those of Dry (1928–29), whose rats were brown (agouti).

At birth, pigment could be detected only in the eyes. On the 1st or 2nd day after birth a bluish discoloration of the skin appeared everywhere but on the midventer, the crown of the head and a thin stripe down the spine. Fur growth over the blue areas followed within a day. The unpigmented venter, crown and spine regions darkened on the 8th day (except belly areas where white fur later grew in some rats), so that by 12 days of age the fur was dark and glossy, but a little shorter in those places where the pigment had appeared last. On the 14th or 15th day the eyes opened. The whole skin remained dark until the 17th or 18th day, when the color began to fade on the crown, then on the back of the neck and so progressively disappeared until at 21 to 22 days of age all of the skin had reverted to its initial pink color. Removal of fur by clipping at this time was not followed by immediate regrowth, as it was while the skin was dark, and no growth occurred until after the reappearance of pigment on the 28th to 35th day. Fig. 2 (Plate I) shows the pale skin of a rat 28 days old, while Fig. 3 shows the same rat a week later (35 days of age). The rat was not reclipped between these pictures; fur had just started to grow on the side and had not yet begun over the pigment on the back.

The second cycle (G_2) reached the stage shown in Fig. 3 at approximately the same time (35 to 37 days of age) in all the rats. After that it slowed down, more in some rats than in others, so that it reached the crown and tail base at ages ranging from 44 to 58 days. Variation between different litters, rather than between members of the same litter, accounted for this spread in the time required to complete G_2.

The third wave (G_3) began low on the side on the 50th day or somewhat later. Fig. 4, again the same rat, but at 59 days, shows G_3

half way up the side while G_2 is still on the crown. Fig. 10 (Plate II), of a different rat, 61 days old, illustrates the same stage but from the side. There is one day's growth of fur up to the narrow stripe of blue skin which represents the most advanced position of G_3 at this time.

The progress of this third wave (G_3) was much slower than that of the second, and proceeded from 1 band of growth to another, rather than covering the whole trunk almost simultaneously. It did not reach the spine until the 59th to 70th day, and was not seen on the crown and tail base until the 90th to 125th day. The subsequent waves were even more retarded and, while still remaining recognizable, tended to break up into spots and patches. G_4 began at about 75 days and was generally completed at 180 days. G_5 began between 100 and 150 days, while G_6 started after 150 and G_7 sometime after 180 days. Fig. 5, the same rat as in Figs. 2–4, but now 114 days old, shows G_3 on the head, G_4 touching the spine in one place only, and G_5 just visible low on the side.

Figs. 6 and 7 (of another, older, male rat) show the characteristic appearance of the skin in rats over 6 months old. In Fig. 6 (209 days old) clipping disclosed G_5 in the lumbar region and also as a tiny blue spot on the crown. G_6, although not well organized, is identifiable on the back of the neck and in the thoracic region, with two wave fronts which are moving toward the places now covered by G_5. G_7 exists as a fragmentary stripe seen on the forearm and also near the hip.

Fig. 7 shows 2 weeks' growth of fur over the pigment shown in Fig. 6. It will be noted that the fur grew black where the skin had been blue (that is on an actively advancing front), and grey in those places where the skin pigment was fading. In Dry's terminology, the follicles were in the telogen (resting) phase in all unblackened areas of the skin, in the anagen (active) phase where black fur grew, and presumably in or entering the catagen phase where grey hairs were produced. Since the proximal part of fully grown hairs on all the normal rats used in this study was grey, owing to the diminution of pigment production toward the end of the growth phase, it is clear that grey hairs appearing after clipping an untreated rat such as this one are merely the proximal segments of normal hairs. This statement is substantiated by the observation that all these grey hairs had cut ends, while the black hairs all had the customary taper.

In many of the older female rats the 5th and subsequent growth cycles appeared in the same patchy and relatively unorganized way as they did in males of equivalent age. Some females, however, had patterns which, although irregular, were more sharply defined and persisted through the course of several cycles. Figs. 8 and 9, of a female rat almost a year old, illustrate this type of curious pattern, which in this case reversed itself several times at intervals of 3 weeks. G_8 covers the center of the body in Fig. 8 and then forms the perfectly dovetailing region over the hip in Fig. 9, while G_9 now forms the cloverleaf pattern on the shoulder. Persistent patterns such as this one have

been seen only in adult female rats, usually occurring when the greater part of an ordinarily sluggish cycle was being completed rapidly. It would seem that in these particular rats definite cleavage lines existed in the skin, across which pattern reversal occurred. Reversal across longitudinal lines as the earlier waves succeeded each other in rising to the spine were, of course, much more common occurrences in both males and females.

In many of the black rats over a year old the hair cycles became exceedingly sluggish. Often no change in appearance was evident for several months. Hair growth occasionally ceased entirely for long periods, leaving the skin perfectly pink. Such a condition usually was accompanied by other indications of disturbed health, and disappeared with improvement in general condition.

Apart from the exceptional females noted above, little or no sex difference in the progression of successive hair cycles was noted. Male and female rats from the same litter were particularly uniform in this respect. As the third growth period (G_3) began, however, small orange spots appeared on the backs on the male rats, first near the tail, then spreading out to cover most of the back (Figs. 4–7). These spots had no apparent relation to hair color or growth, and were never found in a normal female, although in sick or poisoned females a few were often found near the tail (see Fig. 13) but never widespread over the back. A number of additional rats in our colony, albino, tan and hooded, as well as black, revealed the same sex difference, with orange spots appearing on all males and only on those few females which were obviously sick. According to Dr. Maurice Sullivan (who was kind

---

PLATE I: *Normal black rats.* (Note: Background is ruled off in black into 1 cm. squares to show the relative sizes of the rats.)

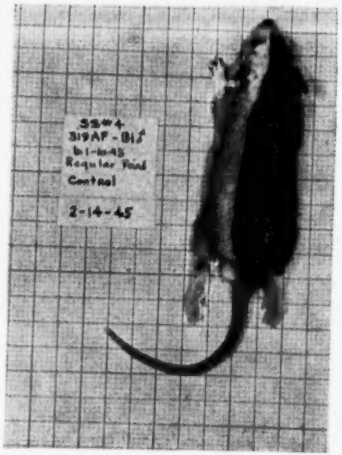
FIGS. 2–5. Successive pictures of untreated male #319 AF; fur removed from left side just before photographing except in Fig. 3. Fig. 2 (28 days old) shows skin entirely free from pigment: growth cycle G_1 is over and G_2 has not yet begun. In Fig. 3 (35 days) G_2 pigment covers all of the body except head and a small region on the tail base. Fig. 4 (59 days) shows G_2 pigment on the crown of the head, while G_3 is rising on the side. Note the superficial orange spots found extensively only in males, which first appear at about this age. In Fig. 5 (114 days) G_3 is closing on crown, G_4 has almost reached spine and G_5 is seen low on side.

FIGS. 6 and 7. Successive pictures of normal male #327 AE, from a litter born 1 day earlier than the rat shown in Plate II. In Fig. 6 (209 days old, fur removed for photograph) G_5 is seen as small blue crown spot and is closing on sacrum, G_6 has reached spine over shoulder, and G_7 is half way up side. Note patchy appearance compared to patterns in rat 114 days old (Fig. 5). Fig. 7 shows fur grown during the next 14 days; the wave fronts have advanced somewhat in the meantime, and black fur has grown over the newly blued parts as well as over the areas appearing blue in Fig. 6. Grey fur has grown where the pigment was fading in Fig. 6, while no fur at all has grown from the other (unblackened) regions of the skin. Note whisker growth (left side) during this period.

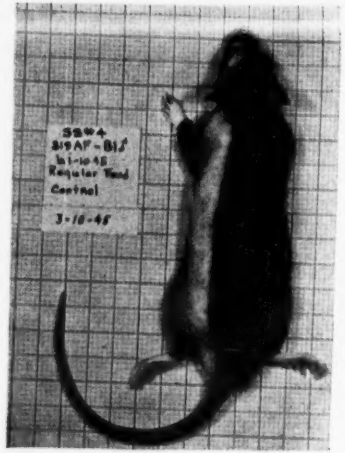
FIGS. 8 and 9. Successive pictures of normal female #373 AB showing sharp pattern reversal in 22 days. Rat clipped just before each photograph. In Fig. 8 (330 days) G_8 covers central region, while in Fig. 9 (352 days) G_9 forms cloverleaf on foreleg and G_8 has shifted toward and is closing on sacrum.

SS#4
319AF-B1♂
b 1-10-45
Regular Food
2-7-45

2


SS#4
319AF-B1♂
b 1-10-45
Regular Food
Control
2-14-45

3


SS#4
319AF-B1♂
b 1-10-45
Regular Food
Control
3-16-45

4

SS#4
319AF-B1♂
b 1-10-45
Regular Food
Control
5-4-45

5

SS#4
327AE-B1♂
b 9-28-44
Control
12-20-44

6

SS#4
327AE-B1♂
b 9-28-44
Control
1-3-45

7

SS#6
372AB-B1♀
b 2-20-45
Control
8-15-44

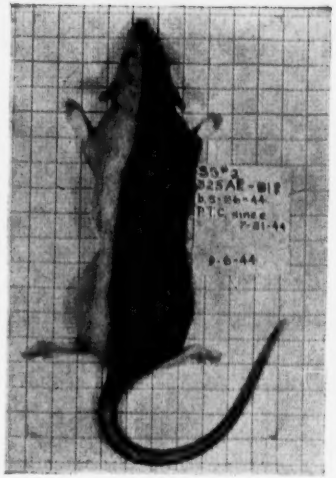
8

SS#6
372AB-B1♀
b 2-20-45
Control
9-6-44

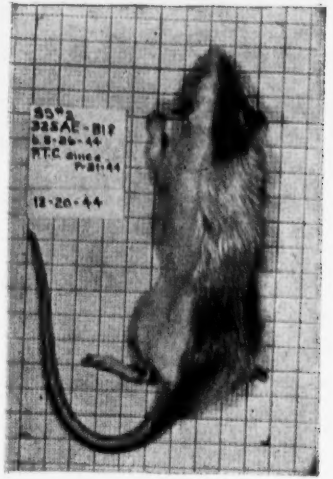
9

10

11

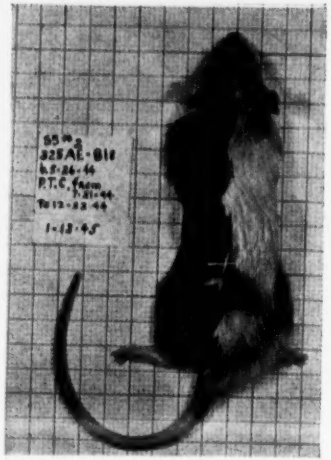


12



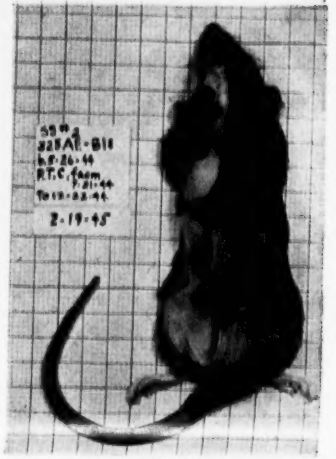
13

14



15

16



17

enough to examine one of the rats), these spots, which are external and can be dislodged with gentle scraping, are composed of oxidized lipoid material extruded from the sebaceous glands. They are apparently a less aggravated form of the brown crusts found by Sullivan and Nicholls (1942) in biotin deficient rats.

Removing the fur from a rat under 200 days old usually revealed 2 or more distinct regions of active hair growth, which showed up in the skin as sharply defined blue-black bands or regions, and whose connection with a particular growth cycle could be traced or even inferred from the age of the rat. In rats over 200 days old the patterns of skin pigment as disclosed by clipping off the fur were usually rather spotty and appeared in a less orderly manner. Generally much less skin pigment was visible at any one time in an old rat than a young one; old rats never had the greater part of the body covered with growing fur (and the accompanying darkened skin) as young rats did.[5] Organized and widespread areas of fur growth seemed to persist much later in life in females than in males, but our observations as to this difference are still too limited to make possible a definite statement on this point.

RESULTS II—POISONED RATS

A. *Thiourea.* Thiourea was administered to 5 rats incorporated in their food, first at 0.1 per cent and later increased to 0.5 and again

[5] An exception to this statement has recently been found in females of our active breeding stock. Preliminary observations on these rats indicate that pregnancy and lactation interrupt the normal cycles so that hair replacement occurs for the most part during the period between weaning of the litter and the subsequent pregnancy. Clipping at this time has revealed pigment and active hair growth over very extensive areas of the body.

PLATE II: *Black rat poisoned with phenyl thiourea*

FIGS. 10–17. Successive pictures of poisoned female #325 AE. Fig. 10 (61 days old, 5 days on poison; fur removed 1 day before photographing) shows cycle G_3 rising on side (compare to Fig. 4), with fur growing over earlier pigment but not on blue wave front. In Fig. 11 (73 days, 17 days on poison, 5 days' fur growth) G_3 has covered most of body and regrown fur appears brownish where pigment was seen most recently, greyish where it had been in Fig. 10. (No change is yet evident in the unclipped fur on right side of body, not shown.) Fig. 12 (103 days old, 47 days on poison, 30 days' fur growth on left side) shows fur on clipped side completely grey, while on unclipped side partial replacement of black hairs by grey has occurred, giving "pepper and salt" appearance except on crown and sacral islands. Fig. 13 (208 days old, 152 days on poison) shows maximum greying of unclipped fur attained with this rat. Fur removed from left side to show absence of skin pigment and presence of some orange spots indicative of a slight toxic effect. Fig. 14 (218 days old, 8 days off poison, 10 days' fur growth) shows skin pigment flooding back, to be covered with black fur on the clipped side in Fig. 15 (232 days old, 22 days off poison, 24 days' fur growth) although little change is yet evident in unclipped fur. In Fig. 16 (243 days old, 33 days off poison, 11 days' fur growth) the surge of pigment has slackened so that regrown fur is brown rather than black (compare to Fig. 11) while several black patches are evident in fur on unclipped side. In Fig. 17 (269 days old, 59 days off poison, 26 days' fur growth) fur is again black everywhere except in 1 small patch, and normal pigment cycles have been resumed.

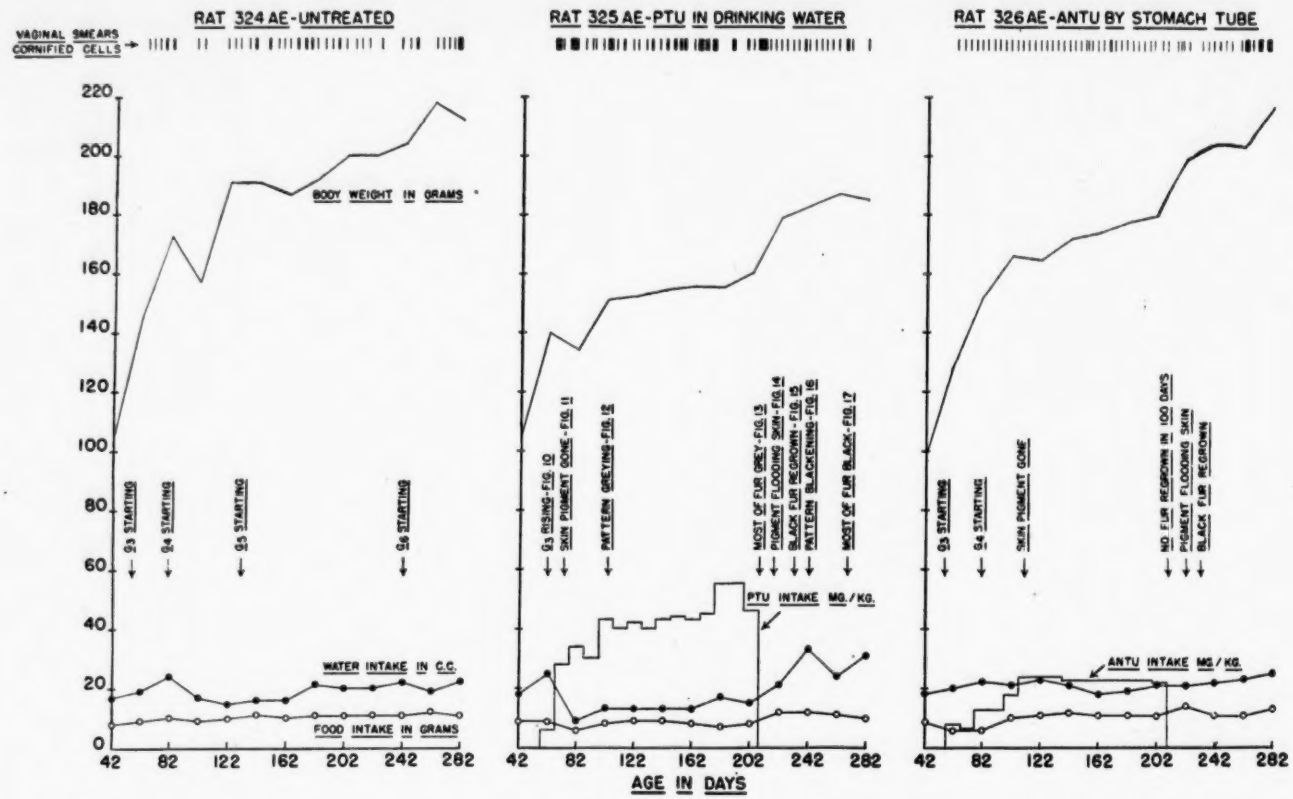


FIG. 1. Records made on 3 female littermate rats, the 1st untreated, the 2nd receiving phenyl thiourea in the drinking water, and the 3rd receiving alpha-naphthyl thiourea as a daily supplement by stomach tube. Each day of full oestrus is indicated by a vertical line in the vaginal smear graph. Body weights at 20-day intervals are connected to give growth curves. Every other 10-day average of food and water intake is plotted, and the poison intakes (10-day averages) are shown in block outlines. (Color photographs of rat #325 AE appear in Figs. 10-17.)

to 1.0 per cent. All 5 of these rats died of chronic poisoning after receiving the drug for periods ranging from 28 to 98 days. Poison intakes reached levels as high as 300 to 450 mg./kg. per day (10-day averages) but dropped off before death as loss of appetite decreased the amount of food eaten. Local edema in the extremities was occasionally observed. The hair cycles continued to be essentially normal and the fur remained black. When given thiourea in their drinking water (at concentrations as high as 0.5 per cent), 4 rats remained in almost perfect health, although their weights leveled off 50 to 100 grams below normal. Their average thiourea intakes were about 200 mg./kg. daily. The hair cycles continued normally and the females had normal oestrous cycles, as shown by vaginal smears.

B. *Phenyl Thiourea.* The experiments with this substance are a continuation of earlier work done in this laboratory, which was reported by Richter and Clisby (1941).

Phenyl thiourea was not so well tolerated as thiourea, the highest average intake being 90 mg./kg. in food and 55 mg./kg. in water, with most rats ingesting nearer 25 to 40 mg./kg. daily. All the rats receiving it in their food (at 0.1%) died of chronic poisoning in from 34 to 82 days. Those receiving it in water (0.05%) remained in better health, and were all alive when the drug was withdrawn (after 130 or 152 days). A typical record of body weight, vaginal smears, and average intakes of food, water and poison for a rat (#325 AE) receiving phenyl thiourea is shown in Fig. 1, together with the corresponding record of an untreated female littermate. The control rat (#324 AE) is typical of other normal rats except for the break in its body weight curve at 102 days of age, the result of a brief illness from which a rapid recovery was made. The distaste of rat #325 AE for increasing concentrations of phenyl thiourea in its drinking water is indicated by the sharp drop in water consumption at the onset of poisoning and the equally rapid rise when the poison was withdrawn. After a slight initial drop, this rat's weight was maintained about 40 grams below normal during the poisoning period, and thereafter rose to a plateau at about 15 grams below normal. Its vaginal smears were irregular during poisoning but changed rapidly after withdrawal to represent regular 4-day cycles.

The hair continued to grow throughout the period of poisoning, but after 21 to 26 days pigment production slowed down so that regrowing fur was brown (Fig. 11). Within another week, regrowth after clipping was entirely of cream-colored fur (Fig. 12) and no further pigment could be detected in the skin for as long as the drug was administered. The effects on the hair showed first on the clipped side of the body, but within a month after beginning the drug (somewhat longer for rats started when 2 months old) enough cream-colored fur had grown on the unclipped side to give a pepper and salt effect everywhere except on the crown and tail base (Fig. 12). Progressively more

intake is plotted, and the poison intakes (10-day averages) are shown in black columns. (Color photographs of the rats appear on Figs. 10-17.)

black hairs fell out on the unclipped side while cream-colored hairs grew; so that after 4 to 5 months almost all the unclipped fur was cream colored, only the crown and rump areas appearing brownish. A small tuft at the proximal end of the tail was the only hair occasionally remaining completely black (Fig. 13).

The changes illustrated for one rat in Figs. 11–13 were typical for rats started on the poison at about two months of age. When treatment began earlier, that is before G_2 had appeared, almost complete greying on both sides of the body was obtained within a month. In one rat, started in the middle of G_2, the crown instead of being the last part to be affected, greyed first, and symmetrically on both clipped and unclipped sides of the body.

After withdrawing the phenyl thiourea the pigment flooded back over almost all of the clipped side in 8 days (Fig. 14),[6] to be covered within 21 days by a new growth of glossy black fur (Fig. 15). No change on the unclipped side was, however, evident until about a month after withdrawal of the poison, when several large black areas had appeared. At this time clipping showed that the flood of pigment was slacking down so that clipped fur was replaced by a rather brown regrowth (Fig. 16, compare to Fig. 11). By $2\frac{1}{2}$ or 3 months after withdrawal all the fur on the body (except sometimes a small sacral patch) was again of normal black color (Fig. 17) and the pigment in the exposed skin was normally distributed in the somewhat irregular pattern characteristic of rats of this age (9 to 10 months).

C. *Alpha-Naphthyl Thiourea.* ANTU was better tolerated in food than either thiourea or phenyl thiourea, although after extended administration deficiency symptoms ("spectacle" eyes and porphyrin deposits around eyes and nose) appeared. One rat died after 69 days (highest average daily intake 79 mg./kg.) but 3 others survived 143 days on the poison with intakes as high as 420 mg./kg. daily. After 16 to 20 days of poisoning (53 to 59 for older rats) all the pigment had disappeared from the skin and scarcely any regrowth of fur occurred thereafter so long as the poison was continued. In all but one rat a few scattered hairs, black, though very downy, grew from time to time. There was, however, no pattern regrowth, nor was greying observed in the unclipped fur, although rusty patches were seen, particularly over the hips, after 3 months of poisoning. (This rusting is of frequent if not universal occurrence in old black rats from this colony.) Withdrawal of the drug was followed, as in the case of phenyl thiourea, by a flooding of pigment over the whole body, then the rapid regrowth of a full coat of black fur and a weight gain up to the normal level.

Another group of rats received alpha-naphthyl thiourea as a daily

[6] It was noted that flooding pigment such as this (and as also found in very young normal rats) always presented a polka-dotted appearance due to the presence of small unpigmented spots about 1 mm. in diameter uniformly scattered over the blued skin.

supplement by stomach tube. The record of one of these (#326 AE), which was typical, appears in Fig. 1. At levels of 25 to 30 mg./kg. pigment production ceased after 3 weeks and hair regrowth also stopped for the entire period of ANTU administration (174 days). Production of both pigment and fur was resumed within 12 days after withdrawing the poison.

RESULTS III—MICE AND WILD NORWAY RATS

Confirmatory observations were made on black mice of the C-57 strain, obtained through the courtesy of Dr. C. C. Little, and maintained on a diet of Purina fox chow supplemented with lettuce. The normal succession of events in these animals was in every way similar to that in rats, with orderly waves of pigment being followed by the growth of fur in younger mice, while isolated spots of pigment were usually found in the skin of older mice. The orange spots seen on the backs of the male black rats were never observed. Attempts to produce greying in these mice by putting phenyl thiourea in their drinking water (at 0.01 to 0.05%) were unsuccessful. The initial mortality due to the acute toxicity of phenyl thiourea was high, but those mice surviving the first few weeks (all of the males died) were maintained on the drug for 12 months without any sign of greying, even after removal of the fur by clipping. Regrowth of normal colored fur continued throughout the experiment. Whether this failure was inherent in the species or was due to a low intake of the drug cannot be said (the mice limited their water intake to less than a half normal during most of the poisoning, and therefore ingested proportionately less phenyl thiourea than was required to produce greying in rats).

Untreated wild Norway rats also showed patterns similar to those found in the black rats, the skin melanin having exactly the same appearance although it preceded the growth of brown rather than black fur. In one wild rat, reared by a domestic mother, the time of appearance of the first few cycles coincided almost exactly with those found in the domestic members of the litter. The orange spots found on the backs of domestic males were found on all adult wild males, and to a lesser extent on the wild females as well, particularly those whose pigment patterns indicated advanced age.

No chronic poisoning experiments were performed with wild rats.

DISCUSSION

Alterations in the color and growth rate of hair in laboratory animals have been observed under a number of experimental conditions. For instance, hair growth is depressed when cystine or certain other essential amino acids are lacking from the diet (Smuts, Mitchell and Hamilton, 1932; Heard and Lewis, 1938; Pavcek and Baum, 1941) or under conditions of semistarvation (Butcher, 1939). Lack of inositol causes alopecia (Cunha et al, 1943; Nielsen and Black, 1944), which,

as it occurs in patterns, can probably be ascribed to replacement failing to keep up with normal hair loss. The subcutaneous administration of chloral hydrate prevents growth of hair (Butcher, 1943).

Achromotrichia has resulted from diets deficient in metals, such as copper (Gorter, 1934, 1935) and zinc (Stirn, Elvehjem and Hart, 1935), or lacking in various B vitamins (Bakke, Aschehoug and Zbinden, 1930; Ansbacher, 1941; Emerson and Evans, 1941; Martin, 1942; Ralli and Graef, 1943). It has also been produced in localized spots by irradiation with cathode rays (Coolidge, 1925) or with high voltage X rays (Hance and Murphy, 1926; Danneel and Lubnow, 1936). The chronic administration of hydroquinone has produced reversible greying in cats (Oettel, 1936).

Hair growth and color are also sensitive to endocrine disturbances (Emmens, 1942). In particular, adrenalectomy has a marked stimulating effect on hair growth (Butcher, 1941; Ralli and Graef, 1943), which appears to be related to the absence of desoxycorticosterone (Ralli and Graef, 1945), while pituitary growth hormone is essential to continued production of hair (Snow and Whitehead, 1935).

It is not yet clear where the effects of phenyl thiourea and of alpha-naphthyl thiourea belong in relation to these other findings. They are at any rate not like the effects of irradiation, which causes permanent damage to the pigment-forming cells. On the other hand, the flooding of the skin with pigment which follows withdrawal of these compounds is very similar to that produced by adrenalectomy.

The thyroid gland is undoubtedly involved, but it does not seem likely that interference with thyroid hormone will provide the complete answer, as it presumably does for thiouracil-induced changes in the feathers of the brown leghorn capon (Juhn, 1944).[7] For although thiourea and phenyl thiourea affected hair and pigment so differently, all the thyroids of rats sacrificed after several months on either of these drugs showed uniformly the same picture of marked hyperplasia and absence of colloid.

The cessation of pigment production may possibly be caused by specific interference with some enzyme necessary for the formation of melanin. The results of work done *in vitro* with thiourea and various of its derivatives are interesting in this connection. Bernheim and Bernheim (1942) and Paschkis et al (1944) found phenyl thiourea to be an effective inhibitor of tyrosinase *in vitro*, while thiourea itself inhibits this enzyme to a much lesser degree. DuBois and Erway (1946) report that alpha-naphthyl thiourea is almost as effective as phenyl thiourea in this respect. But even if such a differential action could also be assumed to obtain *in vivo*, it still would not explain why hair continues to grow under phenyl thiourea treatment and ceases growth with alpha-naphthyl thiourea.

[7] A similar effect has been produced with phenyl thiourea in one such bird generously made available by Dr. B. H. Willier.

There remains the possibility that in the process of detoxification these 3 drugs react to different degrees with some substance in the body. Thiourea is known to react *in vitro* with cysteine (Toennies, 1937), and we have found in preliminary experiments that a supplement of cystine (0.5% in the stock diet) entirely counteracted the suppression of hair growth by alpha-naphthyl thiourea, resulting in normal growth of black hair. This substance may, therefore, compete with the hair for the available cystine. Additional cystine did not, however, change the picture with phenyl thiourea at all.

The greying produced by phenyl thiourea and the suppression of hair production by alpha-naphthyl thiourea were both very easily reversible, which would indicate rapid removal of both substances from the body. A slight decrease of the dose below a daily level of about 25 mg./kg. was rapidly followed by the respective darkening of the fur or resumption of growth.

It should be remarked that none of these drugs had any effect upon the eye pigment, which remained apparently unchanged in every rat (see Figs. 9–16). Nor did they depress whisker growth, which continued even when all other fur replacement was prevented by alpha-naphthyl thiourea; furthermore, the whiskers continued to be frequently pigmented (at least half a dozen on each rat) under treatment with phenyl thiourea. This distinction between ordinary and sensory hairs also occurs in thallium poisoning (Buschke and Peiser, 1926).

SUMMARY

A study has been made of the normal progressive changes in skin pigmentation and in hair growth and color of black rats, brown wild Norway rats, and black mice, and of how these normal patterns are affected in black rats by the chronic administration of thiourea, phenyl thiourea and alpha-naphthyl thiourea. The observations of other workers as to the normal rhythmic production of visible skin pigment in sharply defined areas of skin, followed by growth of hair only where the pigment has appeared, have been confirmed and extended, and a description given of how the stages of follicular activity can be deduced from the gross appearance of the skin. Thiourea has been found to have no significant effect on the hair growth cycles, at average daily intakes as high as 200 mg./kg. body weight, and no effect on the color of the hair or skin. Phenyl thiourea at average daily levels of 25 mg./kg. or higher did not affect hair growth but inhibited pigment formation for as long as it was administered, thereby causing greying of the hair. When the average daily intake of alpha-naphthyl thiourea amounted to 25 mg./kg. or more, hair growth was almost completely inhibited and the skin remained unpigmented. Withdrawal of both phenyl and alpha-naphthyl thiourea resulted in the rapid reversal of these effects.

REFERENCES

ANSBACHER, S.: *Science* 93: 164. 1941.
BAKKE, A., V. ASCHEHOUG AND C. ZBINDEN: *Compt. rend. Acad. d. sc.* 191: 1157. 1930.
BERNHEIM, F., AND M. L. C. BERNHEIM: *J. Biol. Chem.* 145: 213. 1942.
BUSCHKE, A., AND B. PEISER: *Klin. Wchnschr.* 5: 977. 1926.
BUTCHER, E. O.: *Anat. Rec.* 61: 5. 1934–35.
BUTCHER, E. O.: *J. Nutrition* 17: 151. 1939.
BUTCHER, E. O.: *Proc. Soc. Exper. Biol. & Med.* 48: 120. 1941.
BUTCHER, E. O.: *Am. J. Physiol.* 138: 408. 1943.
BUTCHER, E. O., AND R. A. RICHARDS: *Endocrinology* 25: 787. 1939.
COOLIDGE, W. D.: *Science* 62: 441. 1925.
CUNHA, T. J., S. KIRKWOOD, P. H. PHILLIPS AND G. BOHSTEDT: *Proc. Soc. Exper. Biol. & Med.* 54: 236. 1943.
DANNEEL, R., AND E. LUBNOW: *Biologisches Zentralbl.* 56: 572. 1936.
DRY, F. W.: *J. Genetics* 16: 287. 1926.
DRY, F. W.: *J. Genetics* 20: 131. 1928–29.
DIEKE, S. H., AND C. P. RICHTER: *J. Pharmacol. & Exper. Therap.* 83: 195. 1945.
DIEKE, S. H., AND C. P. RICHTER: *Proc. Soc. Exper. Biol. & Med.* 62: 22. 1946.
DUBOIS, K. P., AND W. F. ERWAY: *J. Biol. Chem.*
EATON, P., AND M. W. EATON: *Science* 86: 354. 1937.
EMERSON, G. A., AND H. M. EVANS: *Proc. Soc. Exper. Biol. & Med.* 46: 655. 1941.
EMMENS, C. W.: *J. Endocrinol.* 3: 64. 1942.
FORSTER, A.: *Arch. f. exper. Path. u. Pharmakol.* 144: 363. 1929.
FRASER, D. A.: *Am. J. Anat.* 47: 55. 1931.
GORTER, F. J.: *Nature*, London. 134: 382. 1934.
GORTER, F. J.: *Nature*, London, 136: 185. 1935.
GYÖRGY, P., C. E. POLING AND Y. SUBBAROW: *J. Biol. Chem.* 132: 789. 1940.
HADDOW, A., L. A. ELSON, E. M. F. ROE, K. M. RUDALL AND G. M. TIMMIS: *Nature*, London 155: 379. 1945.
HANCE, R. T., AND J. B. MURPHY: *J. Exper. Med.* 44: 339. 1926.
HAYDAK, M. H.: *Science* 82: 107. 1935.
HEARD, E. V., AND H. B. LEWIS: *J. Biol. Chem.* 123: 203. 1938.
JUHN, M.: *Endocrinology* 35: 278. 1944.
MARTIN, G. J.: *Proc. Soc. Exper. Biol. & Med.* 51: 353. 1942.
MUSHETT, C. W., AND K. UNNA: *J. Nutrition.* 22: 565. 1941.
NIELSEN, E., AND A. BLACK: *Proc. Soc. Exper. Biol. & Med.* 55: 14. 1944.
OETTEL, H.: *Arch. f. exper. Path. u. Pharmakol.* 183: 319. 1936.
PASCHKIS, K. E., A. CANTAROW, W. M. HART AND A. E. RAKOFF: *Proc. Soc. Exper. Biol. & Med.* 57: 37. 1944.
PAVCEK, P. L., AND H. M. BAUM: *Proc. Soc. Exper. Biol. & Med.* 47: 271. 1941.
RALLI, E. P., AND I. GRAEF: *Endocrinology* 32: 1. 1943.
RALLI, E. P., AND I. GRAEF: *Endocrinology* 37: 252. 1945.
RICHTER, C. P., AND K. H. CLISBY: *Proc. Soc. Exper. Biol. & Med.* 48: 684. 1941.
RICHTER, C. P., AND K. H. CLISBY: *Arch. Path.* 33: 46. 1942.
RICHTER, C. P.: *J.A.M.A.* 129: 927. 1945.
SMUTS, D. B., H. H. MITCHELL AND T. S. HAMILTON: *J. Biol. Chem.* 95: 283. 1932.
SNOW, J. S., AND R. W. WHITEHEAD: *Endocrinology* 19: 88. 1935.
STEINER-WOURLISCH, A.: *Ztschr. f. Zellforsch. u. mikr. Anat.* 2: 453. 1925.
STIRN, F. E., C. A. ELVEHJEM AND E. B. HART: *J. Biol. Chem.* 109: 347. 1935.
STRONG, R. M.: *Arch. Path.* 3: 938. 1927.
SULLIVAN, M., AND J. NICHOLLS: *Arch. Dermat. & Syph.* 45: 295. 1942.
TOENNIES, G.: *J. Biol. Chem.* 120: 297. 1937.
TROTTER, M.: *Am. J. Phys. Anthropol.* 7: 427. 1924.
TROTTER, M.: *Anat. Rec.* 37: 373. 1928.

DIFFERENTIATION OF THE ANTITHYROID ACTION OF THIOURACIL, THIOUREA AND PABA FROM SULFONAMIDES BY IODIDE ADMINISTRATION

COSMO G. MACKENZIE

From the Departments of Biochemistry, Cornell University Medical College, NEW YORK, N. Y., *and the School of Hygiene and Public Health, The Johns Hopkins University* BALTIMORE, MD.

In November, 1941, Mackenzie, Mackenzie and McCollum (1941) reported that sulfaguanidine produced a hyperplastic goitre in the rat. At the same time Richter and Clisby (1942) described similar changes in the thyroids of rats poisoned with phenylthiourea. Shortly thereafter in March, the Mackenzies (1942) demonstrated the goiterogenic activity of four additional sulfonamides, and of thiourea itself. It was thus clear that the activity of phenylthiourea was due to the thiourea moiety rather than to any groupings shared in common with sulfaguanidine; and, that the goiterogenic activity of not one but two classes of compounds had been discovered. In November, 1942, Kennedy (1942) suggested that the hitherto unknown goiterogenic agent in rape seed might be allyl thiourea and reported the goiterogenic activity of this compound and of thiourea. Despite the marked dissimilarity in the chemical structure and properties of the thioureas and the sulfonamides, it has hitherto been impossible to demonstrate any decisive differences in the biochemical reactions responsible for their effects on the thyroid.

In a detailed report on these substances by the Mackenzies (1943) it was shown that both classes of compounds lowered the B.M.R., and on the basis of exhaustive experiments it was concluded that they probably did so by "depressing the functional activity of the thyroid," i.e. "the rate of thyroxin formation." According to this view the "thyroidectomy-changes" in the anterior pituitary and the hyperplastic changes in the thyroid were therefore, at least to a large extent, secondary results of the reduction in thyroxin formation. Similar conclusions were reached in the concurrent report of Astwood, Sullivan, Bissell, and Tyslowitz (1943)

In the paper cited above, the Mackenzies (1943) also discovered that *p*-aminobenzoic acid (PABA) was goiterogenic, a fact subsequently confirmed by Astwood (1943) in his study extending the goiterogenic activity of thiourea to its heterocyclic derivatives in-

Received for publication November 15, 1946.

cluding thiouracil. Depression of the B.M.R. by PABA was first conclusively demonstrated by Gordon, Goldsmith, and Charipper (1945). Berman (1945) reported a favorable response to PABA in patients with Graves' disease.

The thyroid inhibition hypothesis advanced by the Mackenzies and Astwood and collaborators found early confirmation in the work of Keston, Goldsmith, Gordon, and Charipper (1944), and Franklin, Lerner, and Chaikoff (1944) who showed that the thioureas prevented the *in vivo* formation of diiodotyrosine and thyroxin from radioactive iodine. *In vitro* experiments by Franklin, Chaikoff and Lerner (1944) on thyroid tissue slices with thioureas, sulfonamides and PABA revealed no differences in the mode of action of these compounds. None of them prevented the penetration of radioiodide into the tissue slices; all of them prevented the iodination of tyrosine. Astwood and Bissell (1944) were unable to distinguish between the effects of thiouracil and sulfadiazine in reducing the iodine content of the thyroid gland of the rat.

After the completion of the work described in this paper, De Robertis and Grasso (1946) reported that thiourea inhibited the staining reaction of the thyroid cells for peroxidase, in confirmation of Dempsey's (1944) earlier observation, while sulfanilamide was without effect. This was correlated with the previous report of De Robertis and Goncalves (1945) that thiourea, but not sulfanilamide, reduces the redox potential of thyroid epithelial cells from +0.05 to −0.20. It should be noted in this connection that the attempt of Glock (1946) to isolate a peroxidase from thyroid tissue met with failure.

The observation of the Mackenzies (1943) that iodide may actually potentiate the effect of sulfaguanidine suggested early in our work that this might serve to differentiate the action of sulfaguanidine from thiourea. Thiourea is a strong reducing agent, a resonating sulfhydryl compound, and consequently it readily reduces iodine to iodide, a reaction studied in detail by Werner (1912). It seemed possible therefore that the antithyroid action of thiourea might be due to the prevention of the formation of appreciable amounts of iodine in the thyroid gland. If this were so, the antithyroid action of thiourea should be inhibited by increasing the iodide intake and thus increasing the formation of iodine by a mass action, providing of course that the thyroid was not already being presented with more iodide than it could oxidize. *On a low but adequate iodide intake* then, increasing the iodide level would have an opposite effect on the response to thiourea than had been observed with sulfaguanidine.

Furthermore the relatively slight though definite thyroid enlargement obtained with 3 per cent PABA on our stock diet (Mackenzie and Mackenzie, 1943) suggested that the response to this compound was being inhibited by iodine and that it also should be tested on a low iodine diet.

Our work on this problem was interrupted by the war. Meanwhile, Astwood and co-workers (1943) had reported that nontoxic levels of iodide had no effect on the thyroid hyperplasia produced by thiourea or sulfaguanidine. On the other hand, when a high level of KI was fed, one sufficiently large to inhibit growth, the hyperplastic response to both thiourea and sulfaguanidine was slightly reduced. The authors concluded that the latter results were probably due to the toxic action of the KI and that in their experiments it was questionable that iodide exerted any specific effect on the thyroid.

Since the results with sulfaguanidine were at variance with our finding that iodide potentiated the response to this drug (Mackenzie and Mackenzie, 1943), our comparative study of the effect of iodide on antithyroid compounds was resumed at the end of the war, and enlarged to include thiouracil. As will be shown in this paper the investigation resulted in the differentiation of the effect on the thyroid of sulfonamides from thiouracil, thiourea, and PABA.

Following the completion of this work there appeared the reports of McGinty and Sharpe (1946), and of Astwood (1945), that iodide decreased the thyroid enlargement produced by thiouracil. This is in accord with our findings.

METHODS

Diet. The purified diet used in this experiment contained the following constituents in parts per kg.; sucrose 560, casein (acid extracted) 200, dried brewers yeast 100, lard 80, salt mixture (McCollum No. 51) 60, and 26 drops of haliver oil fortified with viosterol. The rats fed this diet received in addition 5 mg. of α-tocopherol in 0.05 cc. of peanut oil[1] once a week and distilled water *ad libitum.*

Sulfaguanidine, PABA, and the highest level of thiouracil were mixed in the complete diet. The lower levels of thiouracil were rubbed up in a few gm. of yeast and sucrose, and then thoroughly distributed through the yeast-sucrose fraction. The addition of 500 mg. of sodium iodide per kg. of diet was made by mixing the iodide with the McCollum salt mixture. Incorporation of the lower levels of sodium iodide, 1.0 and 10 mg. per kg. of diet, was effected by dissolving appropriate quantities of the salt in 95 per cent ethanol, and evaporating 10 cc. of these solutions at room temperature for one hour on 200 gm. of casein spread out in a pyrex dish.

Previous analysis had shown that the basal diet contained 37 μg. of iodine per 100 gm. (Mackenzie and Mackenzie, 1943). The addition of the three levels of sodium iodide indicated above raised the iodine content of the diet from 37 μg. per 100 gm. to 122, 887, and 42,537 μg. per 100 gm. Thus, on the enriched diets the iodine intake of the rats was increased approximately 3.3, 24, and 1,150 times respectively.

Experimental Animals. Immature male and female rats for these experiments were obtained from the McCollum stock colony. In each experiment, litter mate rats were evenly distributed throughout the groups receiving different levels of iodide, and as nearly as possible this distribution was equalized with respect to the weight and sex of the rats within a litter. The

[1] Kindly supplied by Merck and Co., Rahway, N. J.

average initial weight of the animals in any one experiment varied from 40 to 56 gm.

Each experiment was of 13 or 14 days' duration. During this time growth was good even on the high iodide diet. For example, on 0.0125 per cent thiouracil the average weight gain was 45 gm. and on the same diet plus 50 mg. of NaI per 100 gm. the average gain was 47 gm.

Thyroid Weight and Histology. The thyroids were weighed on a torsion balance and the weights expressed in mg. per 100 gm. of body weight. The standard error of the mean for small samples was calculated for each group. The significance of the difference between means was determined by calculating t according to Fisher (1932).

One lobe of the thyroid gland from each rat was fixed in Vandegrift's (1942) fluid and the sections stained with hematoxylin and eosin. Each section was examined carefully several times to estimate the height of the epithelial cells and the amount of colloid in the follicles. The epithelium was then scored on the basis of cell types as follows: squamous or low cuboidal 0, mixed squamous and cuboidal 15, cuboidal 25, mixed cuboidal and columnar 50, columnar 100. The colloid was estimated as the per cent of that found in a normal resting thyroid, and assigned an inverse value as follows: 100% = 0, 75% = 25, 50% = 50, 25% = 75, 0 to 10% = 100.

The values thus obtained for epithelium and colloid were added and divided by 2 to obtain a final figure that expressed the degree of thyroid hyperplasia.[2] For an inactive resting gland this figure is 0; for a gland whose follicles are comprised of columnar cells and contain little or no colloid the figure is 100.

RESULTS

The iodine content of the purified diet used in these experiments, 37 μg. per 100 gm., met the normal requirements of the McCollum rat during the 2 week test period as was shown by the failure of added NaI to modify the size or histology of the thyroid (Table I). Remington and Remington (1937) have set the minimum iodine requirements of rats on a commercial stock diet at between 7 and 27 μg. per 100 gm. of diet. The highest level of NaI employed in our experiments, 50 mg. per 100 gm. of diet, did not inhibit growth.

Thioureas and Thyroid Weight. The levels of thiouracil employed were 0.005, 0.0125, and 0.05 per cent; the level of thiourea used was 0.1 per cent. These concentrations were selected to give thyroid responses ranging from a relatively small one to one that approached, but did not reach, the maximum attainable in 2 weeks with thiourea or sulfaguanidine (Mackenzie and Mackenzie, 1943). In this way increases in thyroid weight of approximately 35, 80, 110, and 155 per cent were obtained on the basal diet without added iodide. The mean thyroid weights in mg. per 100 gm. of body weight are shown in Table I. Superimposing increased amounts of iodine in the form of NaI inhibited this response in all cases. The increases in thyroid

[2] The term hyperplasia is used to designate the microscopic changes associated with histological stimulation of the thyroid gland, i.e. loss of colloid, increase in the height of epithelial cells, invagination of the epithelium.

weight were then approximately 0, 38, 41, and 77 per cent respectively. For each level of the antithyroid drugs used the maximum inhibition obtainable occurred with the lowest level of NaI employed.

For 0.005 per cent thiouracil an inhibition approaching 100 per cent was obtained with 1.0 mg. of NaI per 100 gm. of diet. On the higher levels of thiouracil, 0.1 mg. of NaI was tested and found to produce approximately a 50 per cent inhibition in goiterogenic ac-

TABLE I. INHIBITORY EFFECT OF IODIDE ON THE RESPONSE OF YOUNG RATS TO THIOURACIL AND THIOUREA DURING A TWO WEEK TEST PERIOD

No. of rats	Drug	NaI mg. per 100 gm. of diet	Thyroid weight mg. per 100 gm.	t value[1]	Thyroid hyperplasia[2]
7	Thiouracil 0.05%	0	25.5 ± 1.89		100
6	0.05%	0.1	17.7 ± 1.43	3.29	100
6	0.05%	1.0	18.4 ± 1.24	3.11	85
7	0.05%	50	17.0 ± 1.29	3.06	35
8	Thiourea 0.1%	0	21.2 ± 1.19		
8	0.1%	50	14.1 ± 0.77	2.98	
8	Thiouracil 0.0125%	0	17.8 ± 1.34		90
8	0.0125%	0.1	13.8 ± 0.59	2.72	79
8	0.0125%	1.0	13.1 ± 1.18	2.64	49
8	0.0125%	50	14.4 ± 0.62	2.30	15
10	Thiouracil 0.005%	0	13.3 ± 0.44		41
10	0.005%	1.0	10.3 ± 0.51	4.46	18
9	None	0	9.8 ± 1.25		1
4	None	1.0			
7	None	50	10.0 ± 0.33		2

[1] Calculated for the mean thyroid weight of each iodide group and the corresponding control group.

[2] 0 = normal thyroid, 100 = maximum hyperplasia.

tivity. Increasing the iodide concentration even to 50 mg. per 100 gm. did not further limit the increase in thyroid weight.

Oversight of the relationship between iodide intake and the thyroid weight increase effected by thiouracil may lead to conflicting results when antithyroid compounds are assayed on diets differing in their iodine content. Also the estimation of the thyroxin content of materials, such as iodinated casein or desiccated thyroid, on the basis of their effectiveness in preventing thiouracil goitre may be in serious error because of nonthyroxin iodine contaminants if the assay diet is low in iodide.

Thiouracil and Thyroid Histology. Reduction in the size of the goitre produced by thiouracil was not the only effect of iodide. Histological examination of the glands revealed that the addition of iodide, in excess of the amount producing maximum inhibition of

thyroid weight, profoundly modified the histological response to thiouracil. This was surprising since Astwood and co-workers (1943) had reported that nontoxic levels of iodide had no effect on the degree of hyperplasia produced by thiourea.

On the basal diet alone, 0.05 per cent thiouracil produced a maximum hyperplastic response in all of the glands. This picture has been previously described in detail by us (Mackenzie and Mackenzie, 1943). The epithelial cells are exclusively columnar in nature; the lumina are either obliterated or consist of tortuous slits containing occasional bits of vesicular colloid. The follicles are no longer round but are irregular in shape as a result of invagination and the pressure of surrounding follicles. In size they appear to be smaller than normal suggesting that follicular multiplication may have occurred (Fig. 2). The addition of 0.1 mg. of NaI to the 0.05 per cent thiouracil diet, although reducing the response in thyroid weight from 25.5 to 17.7 mg. per 100 gm., did not change this picture.

With the addition of 1.0 mg. of NaI per 100 gm. of diet, the average histological response was modified, although two of the six glands showed no change. One, on the other hand, contained mixed columnar and cuboidal epithelial cells, comprising large follicles that were only slightly involuted. Vacuolated colloid was present in this, and in the remaining three glands. In the latter cases, however, the epithelium was entirely columnar and the follicles were either small or normal in size. Consequently, the primary effect of the added iodide was to increase the amount of colloid found in the average gland. A representative section is illustrated in Fig. 3.

The effect of 50 mg. of NaI was much more pronounced. While the glands from different rats, and even different areas of the same gland, varied with respect to the height of the epithelium, the size and shape of the follicles, and the amount of colloid, the general picture was one of a gland composed about equally of enlarged follicles lined with cuboidal or occasionally low cuboidal epithelium and normal-sized follicles lined with columnar epithelium (Fig. 4). Most of the follicles were filled with colloid, but in some instances only one half to two thirds of the normal amount was present. In many of them the colloid contained free epithelial cells with pycnotic nuclei, cells that had extruded their nuclei, and naked nuclei.

FIG. 1. The resting thryoid gland of a young rat fed a basal diet containing 37μg. of iodine per 100 gm. for 2 weeks (×600).

FIG. 2. The extreme thyroid hyperplasia produced by the administration of the basal diet plus 0.05 per cent thiouracil for two weeks (×600). The irregular shaped follicles are lined exclusively with columnar cells. The invagination of the epithelium has either obliterated the lumina, or reduced them to small slits devoid of colloid. This picture is not changed when 0.1 mg. of NaI per 100 gm. is added to the thiouracil diet although the size of the gland is reduced.

FIG. 3. The effect of 1.0 mg. of NaI per 100 gm. of diet on the response to 0.05 per cent thiouracil (×600). An appreciable amount of vacuolated colloid is present in many

FIG. 1

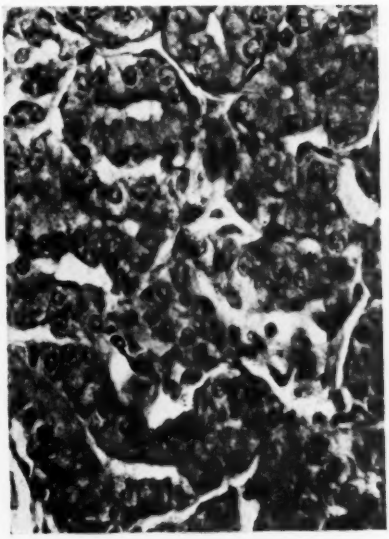


FIG. 2

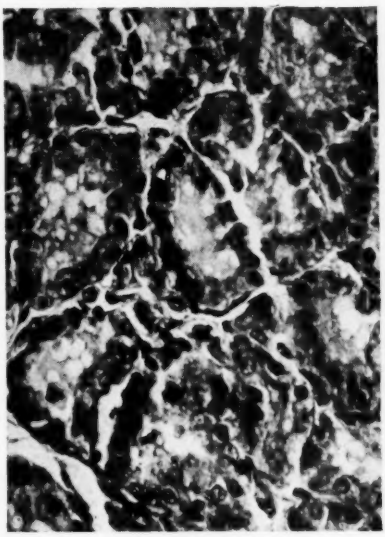


FIG. 3

FIG. 4

follicles. The height of the epithelial cells is reduced though they are still predominantly columnar in character.

FIG. 4. This figure shows one of the large colloid filled follicles observed when 50 mg. of NaI is added to each 100 gm. of the 0.05 per cent thiouracil diet (×600). Side by side with these large follicles are smaller ones comprised of columnar epithelium and vacuolated colloid.

This histological response stands in marked contrast to the one that obtains when either 0.1 mg. or no NaI is added to the basal diet. The average degree of hyperplasia with 0.05 per cent thiouracil and the several levels of iodide employed is given in Table I.

The effect of 0.0125 per cent thiouracil without added iodide differed from 0.05 per cent thiouracil only in that most of the glands contained a small but definite amount of colloid (Fig. 6), and one of them contained columnar epithlium mixed with the cuboidal type.

On a 0.0125 per cent thiouracil diet, to which 0.1 mg. of NaI per 100 gm. had been added, the glands contained approximately one quarter of the full complement of colloid, and in two of eight glands cuboidal cells were found in addition to the columnar type. For the number of animals involved these differences from the diet without added iodide cannot be viewed as significant.

The addition of 1.0 mg. of NaI, however, definitely restricted the hyperplasia. Six of the eight glands contained cuboidal and columnar epithelium in about equal amounts. The nuclei were smaller and their chromatin content was more compact than in the thyroids of non-iodized rats. In the thyroids of the other two rats in this series the epithelium was entirely columnar. The size of the follicles ranged from small, through medium, to large with most of them falling in the last two categories. Approximately 75 per cent of the normal amount of colloid was present (Fig. 7). In the follicles of some glands, dead and dying cells were observed in the colloid.

Even greater depression of thiouracil hyperplasia occurred on the 50 mg. level of NaI. The average gland contained large colloid-filled follicles lined with cuboidal epithelium. In general, the thyroid picture under these conditions bordered on colloid goitre, a colloid goitre produced without passing through Marine's cycle of hyperplasia and involution (Fig. 8).

Columnar epithelial cells were observed in one gland, and low cuboidal or even squamous epithelial cells in two others. In a few follicles large cavities appeared in the center or on the periphery of the colloid. Comparative scores for the degree of hyperplasia observed with this level of thiouracil and different levels of iodide are given in Table I.

Five thousandths of a per cent thiouracil, a level which increased

---

FIG. 5. Low power magnification of the normal resting thyroid gland of a young rat fed the basal diet for 2 weeks (×150).

FIG. 6. Thyroid of rat fed 0.0125 per cent thiouracil for 2 weeks (×150). Except for small amounts of colloid in some follicles the hyperplasia is maximal. The addition of 0.1 mg. of NaI per 100 gm. of diet while reducing the size of the gland did not significantly modify the hyperplasia produced by 0.0125 per cent thiouracil.

FIG. 7. Thyroid of a rat receiving the same treatment but with 1.0 mg. of NaI per 100 gm. of diet (×150). The majority of the follicles contain vacuolated colloid, and they are more nearly normal in shape. In some instances the colloid contains dead and dying epithelial cells. The epithelium is a mixture of cuboidal and columnar cells.

FIG. 8. Nearly complete suppression of the hyperplastic response to 0.0125 per cent

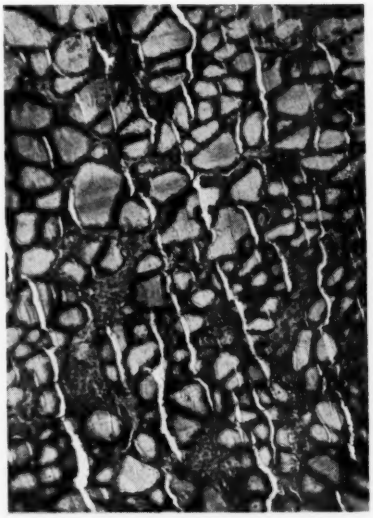


FIG. 5

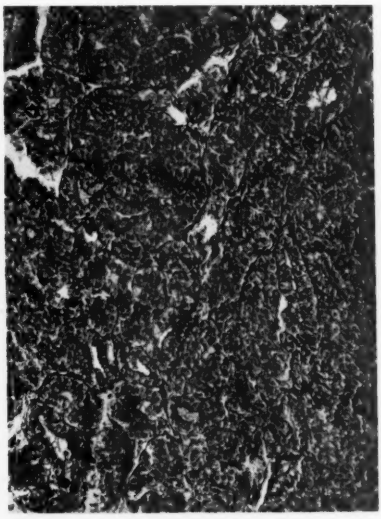


FIG. 6

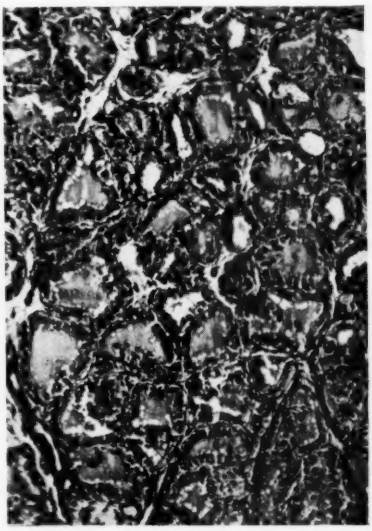


FIG. 7

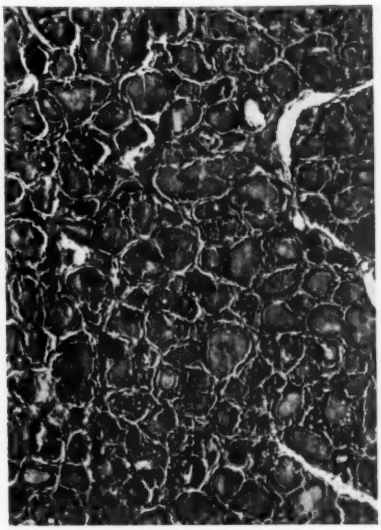


FIG. 8

thiouracil effected by the addition of 50 mg. of NaI to 100 gm. of the diet (×150). Practically all of the follicles are filled with colloid. Much of the epithelium is of a low cuboidal or squamous character, though a higher, even columnar, cell type is found in a few follicles. The thyroids in this series were 40 per cent heavier than the normal and hence represent colloid goiters.

the weight of the thyroid 35 per cent, only slightly reduced the size of the follicles, or their colloid content although some cavities appeared in the colloid. Nevertheless, the epithelium was increased in height. In five of the glands it was of the columnar type, and in the other five a mixture of cuboidal and columnar cells. The addition to the diet of 1.0 mg. of NaI, while restoring the gland to approximately normal weight, only partially inhibited the epithelial hypertrophy. In

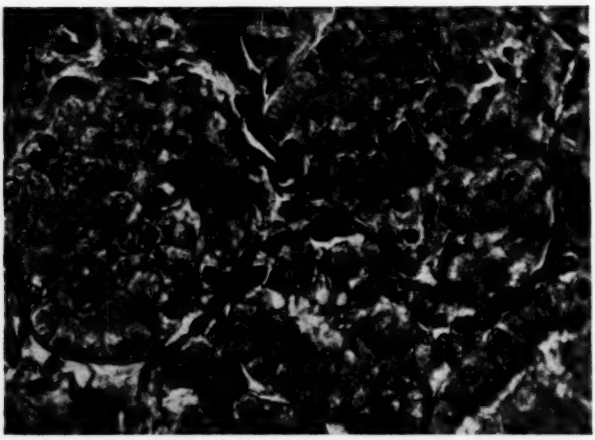


FIG. 9. Typical section through the thyroid gland of a rat receiving 2 per cent sulfaguanidine plus 50 mg. of NaI per 100 gm. of diet for 2 weeks (×600). Note the extreme height of the cells and the invagination of the epithelium. The small lumina are essentially devoid of colloid. Compare this picture with the one obtained when the same level of iodide is added to a diet containing 0.05 per cent thiouracil (Fig. 4).

six thyroids the cells were distinctly cuboidal, as compared to the nearly squamous epithelium of the normal gland; and, in the remainder they were both columnar and cuboidal.

Sulfaguanidine. Sulfaguanidine at a 2 per cent level increased the thyroid weight of young rats by approximately 150 per cent in 2 weeks. This response was comparable to that effected by 0.05 per cent thiouracil. In striking contrast to the latter compound, the addition of NaI to the sulfaguanidine diet in amounts up to 50 mg. per 100 gm. did not inhibit the increase in thyroid weight.

In all of the thyroids of rats receiving 2 per cent sulfaguanidine without iodide the epithelium was of the columnar type. An appreciable amount of colloid was present in the thyroids of two animals. With added amounts of iodide less colloid was found, and on the highest level it was absent from all of the glands (Fig. 9).

To produce a smaller thyroid response, 0.75 per cent of sulfaguanidine was used. Sodium iodide at a concentration of 1.0 mg. per

100 gm. exerted no effect on the size of the gland, but slightly increased the hyperplasia.

The experiment was repeated using 50 mg. of NaI per 100 gm. of diet. This level of iodide produced a 43 per cent increase in gland weight (Table II). The increase in weight is highly significant as indicated by a t value of 5.3 as compared to 2.9 required for the 0.01 level of significance. The relatively small thyroid size of the rats receiving sulfaguanidine alone need not concern us here, for in this experiment a single batch of the sulfaguanidine diet was divided and the NaI added to one half of it. Once again a small increase in the degree of hyperplasia was associated with the addition of iodide.

TABLE II. SYNERGISTIC EFFECT OF A HIGH LEVEL OF IODIDE ON THE RESPONSE OF YOUNG RATS TO A LOW LEVEL OF SULFAGUANIDINE DURING A TWO WEEK TEST PERIOD

No. of rats	Sulfaguanidine %	NaI mg. per 100 gm. of diet	Thyroid weight mg. per 100 gm.	t value[1]	Thyroid hyperplasia[2]
7	2.0	0	24.8 ± 2.28		91
6	2.0	0.1	30.5 ± 2.81	1.58	94
6	2.0	1.0	27.6 ± 2.90	<1	99
6	2.0	50	27.7 ± 2.15	<1	100
10	0.75	0	12.1 ± 0.47		55
10	0.75	1.0	11.9 ± 0.44	<1	66
9	0.75	0	9.8 ± 0.39		59
9	0.75	50	14.0 ± 0.69	5.3	72
8	0.5	0	12.9 ± 0.69		
8	0.5	50	16.8 ± 0.95	3.28	
8	0	0	9.9 ± 0.44		
8	0	50	9.8 ± 0.19		

[1] Calculated for the mean thyroid weight of each iodide group and the corresponding control group.

[2] 0 = normal thyroid, 100 = maximum hyperplasia.

There is also included in Table II an experiment conducted nearly four years earlier (Mackenzie and Mackenzie, 1943). The addition of 50 mg. of NaI to 100 gm. of the low iodine diet increased the thyroid weight obtained with 0.5 per cent sulfaguanidine by 30 per cent. The calculated t value is 3.28. The mean thyroid weights obtained in the earlier experiments for animals on the purified diet with and without NaI attest to the fact that this diet was not deficient in iodine (Table II).

Astwood and co-workers (1943) reported that iodide delayed the development of thyroid hyperplasia in rats fed sulfaguanidine. As the authors suggest, their results were probably due to the toxic level of iodide added to the drinking water.

Para-Aminobenzoic Acid. Five pairs of rats matched as to litter, sex, and weight were used in an exploratory experiment. One rat in each pair received the purified diet containing 2 per cent PABA, and

the other animal was offered the same diet with 0.1 per cent of NaI added. The thyroids of the rats in the former group averaged 14.6 mg. per 100 gm. of body weight, while those of the rats given iodide in addition to PABA averaged 11.2 mg. per 100 gm. The significance of the mean difference between pairs was tested by Fisher's (1932) method and a t value of 7.4 was obtained. The value required for a 0.01 level of significance under these conditions is 4.6

The effect of iodide was then examined in a larger series of rats. PABA alone increased the thyroid weight by approximately 60 per cent. In the presence of 1.0 mg. of NaI per 100 gm. of diet, the thyroid weight response to PABA was *nihil* (Table III). The thyroid glands of these rats were examined histologically. With PABA alone, the follicles were small, and except in two cases where columnar and cuboidal cells were mixed, they were lined entirely with columnar epithelium. The average colloid content of the glands was approxi-

TABLE III. INHIBITORY EFFECT OF IODIDE ON THE RESPONSE OF YOUNG RATS TO TWO PER CENT PABA DURING A TWO WEEK TEST PERIOD

No. of rats	NaI m . per 100 gm. of diet	Thyroid weight mg. per 100 gm.	t value	Thyroid hyperplasia[1]
5	0	14.6		
5	100	11.2	7.4[2]	
8	0	15.9 ± 1.16		74
8	1.0	10.0 ± 0.51	4.7[3]	44

[1] 0 = normal thyroid, 100 = maximum hyperplasia.
[2] Calculated by Fisher's method for pairs.
[3] Calculated from difference between means.

mately 40 per cent of the normal. As was the case with 0.0125 per cent thiouracil (Table I), added iodide partially suppressed this response. Although half of the thyroids contained only columnar epithelium, in the remainder cuboidal and columnar cells were equally common. The average follicle was larger, and the glands contained approximately 75 per cent of the normal amount of colloid.

DISCUSSION

Abundant evidence has been presented in this paper showing that iodide administration exerts distinct and opposite effects on the *in vivo* response of the rat thyroid to thiouracil and sulfaguanidine. Critical conditions for revealing an effect of iodide were employed. First, the basal diet contained a low level of iodine, albeit one that fulfilled the normal requirements for thyroxin synthesis, 37 μg. per 100 gm. of diet. Second, the concentrations of the antithyroid drugs employed did not completely inhibit the formation of thyroxin as judged by a submaximum compensatory enlargement of the thyroid gland.

The response to thiouracil is affected in two ways by the addition of NaI to the basal diet. A small amount of iodide markedly inhibits the thyroid enlargement produced by thiouracil, but it has little or no effect on the thyroid hyperplasia. Larger amounts of iodide have little or no additional effect on the thyroid weight, but they do suppress the hyperplastic changes that normally accompany the compensatory enlargement of the gland.

As to the first of these phenomena, a 33 per cent increase in thyroid weight in response to thiouracil is prevented by iodide, while larger increases in thyroid weight (80 to 155 per cent) undergo a 50 per cent inhibition upon the addition of NaI to the diet. Maximum inhibition was obtained on the lowest level of the salt employed, 0.1 mg. per 100 gm. of diet (Table I).

The second effect of iodide was revealed when 1.0 mg. of NaI was incorporated in each 100 gm. of diet, and it was more extreme, perhaps maximum, when the level was raised to 50 mg. The morphological response to thiouracil was repressed. Hyperemia was reduced, there was less increase in the height of the epithelial cells, the follicles were larger, and more colloid was present. In fact with the higher level of iodide and 0.0125 per cent thiouracil the picture in some glands resembled colloid goitre. With this difference, however, there was never complete suppression of the epithelial hypertrophy. This histological condition is similar to the one found in Graves' disease following the administration of Lugol's solution. It is known from the work of McGinty and Sharpe (1946) and Astwood (1945) that in these suppressed glands there must have been an increase in inorganic iodine.

With sulfaguanidine, iodide neither reduced the size of the thyroid, nor inhibited the degree of hyperplasia. On the contrary, a high level of iodide actually potentiated the goiterogenic action of low levels of sulfaguanidine, and increased the weight of the gland by 30 to 40 per cent. Furthermore, there was a tendency for iodide under all conditions to decrease the colloid content of the glands of sulfaguanidine-treated rats. This is probably the only condition thus far described in which iodide does not have an inhibitory effect on thyroid hyperplasia.

It may be concluded from the foregoing results that the primary biochemical reactions whereby thiouracil and sulfaguanidine exert their effects on the thyroid are different. If these reactions were equivalent, the addition of iodide, itself an essential component of thyroxin, would modify the response to both compounds in the same way.

Hypotheses concerning the action of the antithyroid agents may be deduced from these experiments. With respect to thiouracil these deductions hinge on whether the reduction in thyroid weight caused by a small amount of iodide was due to a direct action on the pitui-

tary gland or thyrotropic hormone, or whether it resulted from an increase in thyroid hormone synthesis. So far as the author knows there is no evidence that the ingestion of as little as 8 or 10 μg. of NaI daily will suppress either the elaboration or the action of thyrotropin in the rat. Furthermore if such an inhibition were assumed the failure of iodide to exert such an effect in the presence of sulfaguanidine would be difficult to explain. It appears probable therefore that in these experiments added iodide slightly increased thyroxin formation in the presence of thiouracil and thiourea.

Many investigators (Baumann, Metzger, and Marine, 1944; Keston, 1944; Campbell, Landgrebe, and Morgan, 1944; Miller, Roblin, and Astwood, 1945; Calvo and Goemine, 1946) have associated the well known reduction of iodine to iodide by sulfhydryl compounds, shown specifically for thiourea by Werner (1912), and for thiouracil by Miller, Roblin, and Astwood (1945), with antithyroid activity. According to Li (1942) and Harrington (1944) iodide must be oxidized to effect the iodination of tyrosine. Miller, Roblin, and Astwood (1945) and Calvo and Goemine (1946) have shown that this iodination is prevented *in vitro* when thioureas are present, and Keston (1944) has shown that organic iodine is not formed in milk in the presence of a xanthine, xanthine oxidase, peroxidase system when thiourea is added. However, there has been no evidence against the alternative hypothesis that the thioureas, as reducing agents, directly inhibit the iodide oxidizing system (enzymatic or otherwise), and in fact this is the theory proposed by Dempsey (1944) and De Robertis and Grasso (1946) on the basis of their peroxidase studies. The present paper furnishes evidence against this last hypothesis, and presents *in vivo* evidence favoring the view that thioureas exert their antithyroid action by reducing iodine. For if the thioureas inhibit the oxidizing system, be it enzymatic or not, no action of thioureas should be noticed until this system is no longer able to oxidize the small but adequate amount of iodide furnished by the basal diet. Then increasing the level of iodide would have no effect, and judged from the decrease in thyroid weight, this is not the case. If on the other hand thioureas simply competed with iodide for the oxidizing system, the effect of increasing the iodide concentration should not be limited, as it is, while the iodide content of the thyroid is increasing as shown by McGinty and Sharpe (1946) and Astwood (1945). This leaves then the reduction of iodine (or hypoiodite) as fast as it is formed by the oxidation of iodide, as the most probable mechanism responsible for the action of the thioureas.

If this hypothesis is correct, adding iodide would result in increased iodine and hence thyroxin formation until the oxidizing system was saturated with respect to the ion and hence working at a maximum rate. Adding still more iodide would be of no avail. This situation fits the results reported herein. As Dempsey and Astwood

(1944) have shown, the formation of even 1.25 μg. of thyroxin a day would appreciably reduce the size of the thyroid. That the thioureas may also compete with iodide for an oxidizing system is not excluded. Randell (1946) has demonstrated that thiourea is oxidized by a plant peroxidase-peroxide system. However, the hypothesis presented here states that the thioureas are such potent antithyroid agents *primarily* because they can reduce iodine (or hypoiodite) as fast as it is formed. This hypothesis is subject to experimental test by the *in vitro* technique for following diiodotyrosin and thyroxin synthesis in thyroid tissue slices devised by the University of California group (Perlman, Morton, and Chaikoff, 1941; Morton and Chaikoff, 1943).

Sulfaguanidine on the other hand must act in a different way. The addition of iodide appears to exert no mass action effect on the chain of reactions leading to thyroxin synthesis when sulfaguanidine is fed, suggesting the possibility that the sulfonamides inhibit an essential enzyme in this chain of reactions. This is apparent when sulfaguanidine is substituted for thiouracil in the argument applied to the latter compound. Lipmann (1941) has reported the inhibition of horseradish peroxidase by sulfanilamide.

The failure of large amounts of iodide to restrict the epithelial hyperplasia and loss of colloid produced by sulfaguanidine in these experiments may be related to the blocking of thyroid hormone formation by this compound. However, it should be noted that in long term experiments with sulfaguanidine there is some involution of the hyperplastic thyroid even though the B.M.R. is minus 30 (Mackenzie and Mackenzie, 1944).

In vitro experiments in which the iodide concentration is varied in the presence of a constant level of sulfaguanidine would test the enzyme inhibition hypothesis advanced above.

Iodide administration inhibits the goiterogenic and hyperplastic activity of PABA. In this respect PABA resembles not sulfaguanidine but the thioureas. This is not to say however that the latter compounds and PABA exert their effects on the thyroid through identical mechanisms. PABA does not compare with these sulfhydryl compounds as a reducing agent, and the reasoning applied to them cannot be extended to this aromatic amine. If the existence of a peroxide-peroxidase system in the thyroid could be unequivocally demonstrated, one might infer that PABA competed with iodide as a substrate for such a system.

ADDENDUM

In additional experiments on sulfonamides and PABA, the acid-washed casein in the diet was replaced with vitamin-free casein, and Rockland Farm male rats replaced animals of the McCollum stock. Under these conditions the basal diet was slightly goiterogenic (Table IV). The thyroid enlargement caused by PABA was again completely

suppressed by sodium iodide, this time by 0.05 per cent. With sulfanilamide or sulfadiazine, however, the increase in thyroid weight was not significantly reduced in either case by the concomitant administration of iodide. Indeed the small increase in thyroid weight produced by sulfanilamide on the already goiterogenic basal diet was actually augmented by the inclusion of iodide.

These results show that the resistance of sulfaguanidine to iodide prophylaxis is not unique, but is a quality possessed by other, possibly all, sulfonamides. Furthermore, they show that PABA and the sulfonamides are *not in general interchangeable* with respect to their effects on the thyroid of the rat. Since the aniline portion of the molecules of both types of compounds is essential for activity (Mac-

TABLE IV. EFFECT OF 0.05 PER CENT SODIUM IODIDE ON THE RESPONSE OF YOUNG MALE RATS TO PABA AND SULFONAMIDES DURING A TWO WEEK TEST PERIOD

No. of rats	Drug	Thyroid weight mg. per 100 gm.		t value
		No NaI	Added NaI	
13	None	8.4 ± 0.46	6.4 ± 0.27	3.8
20	2% PABA	16.5 ± 0.75	7.3 ± 0.37	11.0
17	1% Sulfanilamide	10.2 ± 0.48	14.9 ± 1.84	2.5
18	0.2% Sulfadiazine	15.5 ± 1.59	14.4 ± 0.84	0.6

kenzie, *et al.* 1943; Astwood, 1943), though aniline itself is not active *in vivo* (Astwood, 1943), an unusual situation obtains. Substituting either of two different radicals, the sulfonamide or the carboxy, on the aniline ring produces molecules not unrelated in structure, both of which reduce the formation of thyroxin, but which exert their effects on the thyroid in some essentially different manner.

CONCLUSIONS

Iodide exerts different and opposite effects on the response of the rat to the antithyroid drugs, thiouracil and sulfaguanidine.

Small amounts of iodide inhibit the goiterogenic action of thiouracil by 50 to 100 per cent, depending on the level of the goiterogen employed. Higher levels of iodide, while not increasing this inhibition, suppress the thyroid hyperplasia.

The goiterogenic effect of low levels of sulfaguanidine is augmented by a high level of iodide. With either low levels of iodide or a high level of sulfaguanidine, this synergistic action is not observed. However, iodide appears to increase the degree of thyroid hyperplasia under all of the conditions studied.

PABA resembles thiouracil rather than sulfaguanidine in so far as both its goiterogenic and hyperplastic actions are inhibited by iodide.

It is concluded that thiouracil, thiourea, and PABA differ from the sulfonamides in the primary biochemical reactions responsible for their effects on the thyroid.

REFERENCES

ASTWOOD, E. B.: *J. Pharmacol. & Exper. Therap.* 78: 79. 1943.
ASTWOOD, E. B.: The Harvey Lectures, Science Press, Lancaster, Penn., 1945.
ASTWOOD, E. B., AND A. BISSELL: *Endocrinology* 34: 282. 1944.
ASTWOOD, E. B., J. SULLIVAN, A. BISSELL AND R. TYSLOWITZ: *Endocrinology* 32: 210. 1943.
BAUMANN, E. J., N. METZGER AND D. MARINE: *Endocrinology* 34: 44. 1944.
BERMAN, L.: *Proc. Soc. Exper. Biol. & Med.* 59: 70. 1945.
CALVO, J., AND J. GOEMINE: *Arch. Biochem.* 10: 531. 1946.
CAMPBELL, D., F. W. LANDGREBE AND T. N. MORGAN: *Lancet* 1: 630. 1944.
DEMPSEY, E. W.: *Endocrinology* 34: 27. 1944.
DEMPSEY, E. W., AND E. B. ASTWOOD: *Endocrinology* 32: 509. 1944.
DE ROBERTIS, E., AND J. M. GONÇALVEZ: *Endocrinology* 36: 245. 1945.
DE ROBERTIS, E., AND E. GRASSO: *Endocrinology* 38: 137. 1946.
FISHER, R. A.: Statistical Methods for Research Workers, Oliver and Boyd, London, 1932.
FRANKLIN, A. L., AND I. L. CHAIKOFF: *J. Biol. Chem.* 152: 295. 1944.
FRANKLIN, A. L., I. L. CHAIKOFF AND S. R. LERNER: *J. Biol. Chem.* 153: 151. 1944.
FRANKLIN, A. L., S. R. LERNER AND I. L. CHAIKOFF: *Endocrinology* 34: 265. 1944.
GLOCK, H. E.: *Nature London* 154: 460. 1946.
GORDON, A. S., E. D. GOLDSMITH AND H. A. CHARIPPER: *Endocrinology* 37: 223. 1945.
HARRINGTON, C. R.: *J. Chem. Soc.* 1: 193. 1944.
KENNEDY, T. H.: *Nature*, London 150: 233. 1942.
KESTON, A. S.: *J. Biol. Chem.* 153: 335.1944.
KESTON, A. S., E. D. GOLDSMITH, A. S. GORDON AND H. A. CHARIPPER: *J. Biol. Chem.* 152: 241. 1944.
LI, C. H.: *J. Am. Chem. Soc.* 64: 1147. 1942.
LIPMANN, F.: *J. Biol. Chem.* 139: 977. 1941.
MACKENZIE, J. B., AND C. G. MACKENZIE: *Federation Proceedings* 1: 122. 1942.
MACKENZIE, C. G., AND J. B. MACKENZIE: *Endocrinology* 32: 185. 1943.
MACKENZIE, J. B., AND C. G. MACKENZIE: *Bull. Johns Hopkins Hosp.* 74: 85. 1944.
MACKENZIE, J. B., C. G. MACKENZIE AND E. V. MCCOLLUM: *Science* 94: 518. 1941.
MCGINTY, D. A., AND E. A. SHARP: *Endocrinology* 39: 74. 1946.
MILLER, W. H., R. O. ROBLIN AND E. B. ASTWOOD: *J. Am. Chem. Soc.* 67: 2201. 1945.
MORTON, M. E., AND I. L. CHAIKOFF: *J. Biol. Chem.* 147: 1. 1943.
PERLMAN, I., M. E. MORTON AND I. L. CHAIKOFF: *J. Biol. Chem.* 139: 449. 1941.
RANDELL, L. O.: *J. Biol. Chem.* 164: 521. 1946.
REMINGTON, R. E., AND J. W. REMINGTON: *J. Nutrition* 15: 539. 1938.
VANDEGRIFT, W. B.: *Bull. Johns Hopkins Hosp.* 71: 98. 1942.
WERNER, E. A.: *J. Chem. Soc.* 101: 2166. 1912.

THE EFFECT OF VAGOTOMY AND OF THORACIC SYMPATHECTOMY ON THE BLOOD GLUCOSE CHANGES IN DOGS GIVEN ALLOXAN

ELVA G. SHIPLEY AND KARL H. BEYER

From the Department of Pharmacology, The Medical Research Division, Sharp and Dohme, Inc.

GLENOLDEN, PA.

SINCE the discovery by Dunn, Sheehan, and McLetchie (1943) that alloxan injections produce necrosis of the pancreatic islet tissue in rabbits, considerable work has been done in an attempt to clarify the mechanism of production of diabetes by alloxan, and the factors responsible for the triphasic curve of blood sugar changes following the injection of alloxan. Many investigators have attributed the blood glucose changes after alloxan to a rapid and direct action of the alloxan on islet tissue, thus causing the cells to liberate preformed insulin. Hughes, Ware, and Young (1944) found that the hypoglycemic action of alloxan could be simulated in the rabbit by the administration of protamine zinc insulin in an amount equivalent to the extractable insulin in the pancreas of the normal animal. Ridout, Ham, and Wrenshall (1944) concluded that the insulin content of the pancreas did not fall until most of the islet beta cells were found to be dead, and that the stimulation of beta cell secretion was an unlikely cause of hypoglycemia, since the islet cells were believed to be dead when the hypoglycemic phase occurred. Gomori and Goldner (1945) clamped blood vessels supplying portions of the pancreas of dogs and found that under such conditions alloxan injections produced necrosis of islets in the unclamped portions, but the occluded portions of the pancreas did not show necrosis. The accumulation of evidence led to acceptance of the theory that alloxan acts directly upon islet cells by way of the blood stream. Dunn, Kirkpatrick, McLetchie, and Telfer (1943) suggested the possibility of excessive stimulation and functional overactivity of the insular tissue, and pointed out the resemblance of the blood glucose changes to those seen in hypothalamic stimulation.

It was shown by LaBarre and Vesselowsky (1933) and by others that vagal stimulation produced a decrease in blood glucose, and Sergeyeva (1938) found that prolonged stimulation of vagi produced an appearance of exhaustion in islet tissue. Sergeyeva's preparations bore a striking resemblance to the pancreatic islets of animals killed

Received for publication December 6, 1946.

or dying in hypoglycemic convulsions, a few hours after alloxan treatment. The results of Shipley (1944) indicated that hypoglycemic convulsions occurred in alloxan-treated adrenalectomized rats as early as 5 minutes after the injection, and that in hypophysectomized rats the lowest blood glucose values were seen 24 hours after alloxan administration.

The investigation reported here was undertaken in an attempt to find the effect of resection of vagi and of sympathetic chains upon the animal's physiologic response to alloxan treatment, and to ascertain whether any of the blood sugar changes were mediated by way of the nerves supplying the pancreas.

MATERIALS AND METHODS

Dogs of mixed breeds were used in this investigation. They were obtained from commercial dealers and were kept in the laboratory on the regular diet of 'Friskies' supplemented with horse meat. All surgical operations were performed under cyclopropane and ether anesthesia and under aseptic conditions. One group of four animals was unoperated; another group was composed of five dogs that had been bilaterally vagotomized supradiaphragmatically and had bilateral resection of the lower thoracic sympathetic chains, involving the removal of five or six ganglia from each chain. The operations were performed in two stages; in the first stage the vagi were cut and the sympathetic chain of one side was resected. After complete recovery from the first operative procedures a second operation was performed to resect the sympathetic chain of the other side. The animals were allowed to recover from the latter operation before they were given alloxan. The third group had both lower thoracic sympathetic chains resected in two-stage operations, and a fourth group had only the vagi cut in a single stage operation. In a fifth group two dogs were sham-operated. They were subjected to all the traumatic effects of the two-stage bilateral resection of the vagi and sympathetics, except the nerves were left intact. When the incisions had healed and the dogs showed a gain in weight they were considered ready to be used for alloxan treatment. The time between the last operation and alloxan injections was usually ten to fifteen days. Food was removed from the dogs 16 to 18 hours before they were to be injected and blood samples were obtained for glucose determinations before alloxan administration. Blood samples were obtained from the ear veins or the cut tip of the tail. Blood glucose determinations were made using the colorimetric method of Reinecke (1942) adapted to the Evelyn photoelectric colorimeter.

The dose of alloxan employed was 75 mg./kg. of body weight. The alloxan was made up in a 5 per cent aqueous solution, and was given as a single injection into the external saphenous or brachial vein. At stated intervals blood samples were obtained for glucose determinations.

RESULTS AND DISCUSSION

Four control intact dogs that were fasted overnight and given alloxan exhibited the phases of the blood sugar curves found by other investigators (table 1). Blood samples were taken before alloxan injection and at 0.25, 0.5, 1, 2, 4, 6, 8, 10, 12, and 24 hours after

THE EFFECT OF VAGOTOMY AND OF THORACIC SYMPATHECTOMY ON THE BLOOD GLUCOSE CHANGES IN DOGS GIVEN ALLOXAN

ELVA G. SHIPLEY AND KARL H. BEYER

From the Department of Pharmacology, The Medical Research Division, Sharp and Dohme, Inc.

GLENOLDEN, PA.

SINCE the discovery by Dunn, Sheehan, and McLetchie (1943) that alloxan injections produce necrosis of the pancreatic islet tissue in rabbits, considerable work has been done in an attempt to clarify the mechanism of production of diabetes by alloxan, and the factors responsible for the triphasic curve of blood sugar changes following the injection of alloxan. Many investigators have attributed the blood glucose changes after alloxan to a rapid and direct action of the alloxan on islet tissue, thus causing the cells to liberate preformed insulin. Hughes, Ware, and Young (1944) found that the hypoglycemic action of alloxan could be simulated in the rabbit by the administration of protamine zinc insulin in an amount equivalent to the extractable insulin in the pancreas of the normal animal. Ridout, Ham, and Wrenshall (1944) concluded that the insulin content of the pancreas did not fall until most of the islet beta cells were found to be dead, and that the stimulation of beta cell secretion was an unlikely cause of hypoglycemia, since the islet cells were believed to be dead when the hypoglycemic phase occurred. Gomori and Goldner (1945) clamped blood vessels supplying portions of the pancreas of dogs and found that under such conditions alloxan injections produced necrosis of islets in the unclamped portions, but the occluded portions of the pancreas did not show necrosis. The accumulation of evidence led to acceptance of the theory that alloxan acts directly upon islet cells by way of the blood stream. Dunn, Kirkpatrick, McLetchie, and Telfer (1943) suggested the possibility of excessive stimulation and functional overactivity of the insular tissue, and pointed out the resemblance of the blood glucose changes to those seen in hypothalamic stimulation.

It was shown by LaBarre and Vesselowsky (1933) and by others that vagal stimulation produced a decrease in blood glucose, and Sergeyeva (1938) found that prolonged stimulation of vagi produced an appearance of exhaustion in islet tissue. Sergeyeva's preparations bore a striking resemblance to the pancreatic islets of animals killed

Received for publication December 6, 1946.

or dying in hypoglycemic convulsions, a few hours after alloxan treatment. The results of Shipley (1944) indicated that hypoglycemic convulsions occurred in alloxan-treated adrenalectomized rats as early as 5 minutes after the injection, and that in hypophysectomized rats the lowest blood glucose values were seen 24 hours after alloxan administration.

The investigation reported here was undertaken in an attempt to find the effect of resection of vagi and of sympathetic chains upon the animal's physiologic response to alloxan treatment, and to ascertain whether any of the blood sugar changes were mediated by way of the nerves supplying the pancreas.

MATERIALS AND METHODS

Dogs of mixed breeds were used in this investigation. They were obtained from commercial dealers and were kept in the laboratory on the regular diet of 'Friskies' supplemented with horse meat. All surgical operations were performed under cyclopropane and ether anesthesia and under aseptic conditions. One group of four animals was unoperated; another group was composed of five dogs that had been bilaterally vagotomized supradiaphragmatically and had bilateral resection of the lower thoracic sympathetic chains, involving the removal of five or six ganglia from each chain. The operations were performed in two stages; in the first stage the vagi were cut and the sympathetic chain of one side was resected. After complete recovery from the first operative procedures a second operation was performed to resect the sympathetic chain of the other side. The animals were allowed to recover from the latter operation before they were given alloxan. The third group had both lower thoracic sympathetic chains resected in two-stage operations, and a fourth group had only the vagi cut in a single stage operation. In a fifth group two dogs were sham-operated. They were subjected to all the traumatic effects of the two-stage bilateral resection of the vagi and sympathetics, except the nerves were left intact. When the incisions had healed and the dogs showed a gain in weight they were considered ready to be used for alloxan treatment. The time between the last operation and alloxan injections was usually ten to fifteen days. Food was removed from the dogs 16 to 18 hours before they were to be injected and blood samples were obtained for glucose determinations before alloxan administration. Blood samples were obtained from the ear veins or the cut tip of the tail. Blood glucose determinations were made using the colorimetric method of Reinecke (1942) adapted to the Evelyn photoelectric colorimeter.

The dose of alloxan employed was 75 mg./kg. of body weight. The alloxan was made up in a 5 per cent aqueous solution, and was given as a single injection into the external saphenous or brachial vein. At stated intervals blood samples were obtained for glucose determinations.

RESULTS AND DISCUSSION

Four control intact dogs that were fasted overnight and given alloxan exhibited the phases of the blood sugar curves found by other investigators (table 1). Blood samples were taken before alloxan injection and at 0.25, 0.5, 1, 2, 4, 6, 8, 10, 12, and 24 hours after

alloxan administration and at various intervals in the days following the first 24 hours. The reported tri-phasic curve includes a slight or moderate rise in blood sugar at 2 to 4 hours after alloxan administration followed by a marked hypoglycemia at 6 to 12 hours after treatment. In addition to these three phases of blood sugar changes described by other investigators the blood glucose values in sixteen of the nineteen dogs of these experiments were lower at 0.25 or 0.5 hour after alloxan treatment than at the fasting level preceding the injection (table 1, figures 1 to 5). This very early drop in blood sugar, which appeared before the transitory hyperglycemic phase,

TABLE 1. BLOOD GLUCOSE VALUES (MG/100 ML.) IN DOGS

No.	Treatment	wt. (kg.)	Hours after alloxan injection												
			0	¼	½	1	2	4	6	8	10	12	24	26–29	48
137	unoperated	9.4	114	73	100	157	260	228	150	65	—	63	133	—	600
138		7.8	96	116	175	180	195	155	68	50	73	150	251	—	480
144		9.6	114	73	83	94	100	87	55	39*	28*	42*	99	—	—
145		8.1	103	105	115	165	188	80	62	60	65	108	78	—	—
147	vagotomy and thoracic	13.3	100	123	106	127	151	191	163	138	131	138	58*	50*	470
143	sympathectomy	10.0	81	58	65	80	108	100	83	73	96	115	45*	53*	243
135		14.9	102	61	125	143	205	215	179	138	92	113	88	256	360
109		7.7	108	95	105	106	176	256	227	173	93	88	54*	127	332
129		5.3	115	69	83	83	153	138	65	35*	90	46*	220	—	291
213	thoracic sympathectomy	10.5	105	92	125	122	168	144	75	64	43	45	136	160	269
219		13.6	113	75	113	128	195	238	204	132	130	134	73	564	596
179		15.2	100	65	80	103	104	150	158	149	85	101	218	—	438
206		16.7	115	85	113	111	165	190	163	116	63	90	150	—	388†
126	vagotomy	10.3	103	48	91	103	145	216	240	225	138	102	58	52	285
127		7.9	98	43	90	130	149	113	50	40	48*	120	330	265	415
139		11.3	99	74	55	102	153	160	120	62	68	91	138	285	360
166		8.9	95	46	71	100	200	188	140	138	73	71	55	85	233
160	sham-operation	11.8	98	81	86	105	149	133	138	108	109	150	253	194	311
161		11.6	110	98	130	150	188	194	165	96	80	89	106	128	401

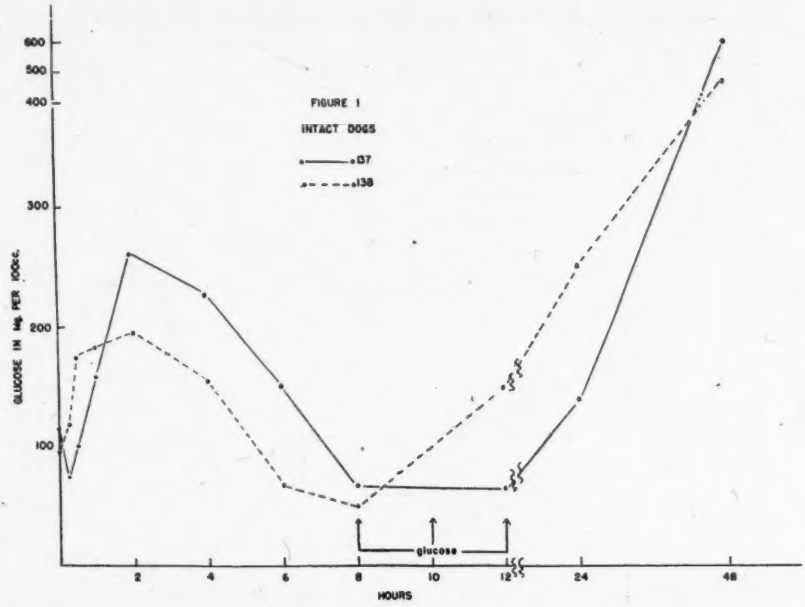
*administration of glucose.
† 72 hr. sample.

could be detected only by frequent sampling of blood glucose. The tetra-phasic blood glucose curves seen in the animals of these experiments suggest a release of insulin within the first few minutes following alloxan injection. This interpretation is supported by the observations of Shipley (1944) that adrenalectomized rats exhibit hypoglycemic convulsions as early as five minutes after alloxan injections.

The lowest blood sugar values in the control intact dogs of the first group were found at 8, 10, or 12 hours after alloxan injection (figure 1). Twenty-four hours after the alloxan treatment the blood sugar was back to the fasting level or above it. Only one animal failed to show a final marked hyperglycemia, yet suffered from toxic effects of the alloxan and lived only five days after the injection. Alloxan has been shown to produce necrosis of proximal tubules in the kidney and it appears probable that extra-pancreatic damage contributed to this animal's death. The animal died overnight and, when found, autolysis had progressed to the extent that tissue study

could afford little information as to the cause of death.

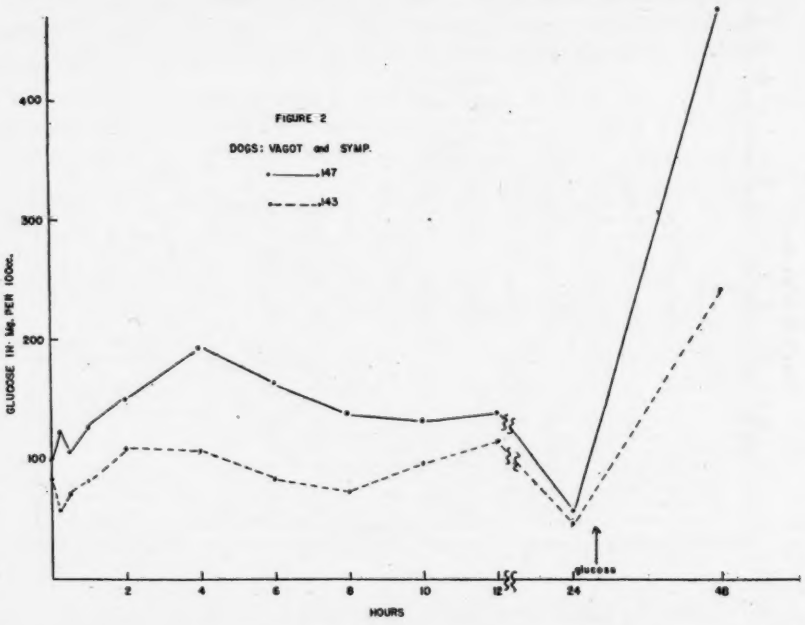
Five dogs with bilateral vagotomy and lower thoracic sympathectomy were used in the next group. Post-operative care was as decribed earlier. Alloxan injections were made in the same manner and the dogs were given the same dose equivalents of alloxan as were given to the intact dogs. The blood glucose changes were similar to



the changes observed in the intact dogs during the first six hours following alloxan treatment. Four of the five dogs were found with hypoglycemic symptoms 24 hours post-injection and three of the four required the administration of glucose to relieve convulsive attacks. Fifty cc. of a 10 per cent glucose solution was administered by intraperitioneal injection at 24 hours after alloxan treatment to the convulsive dogs. Two to four hours later they were again in convulsive hypoglycemia and a second dose of glucose solution was given as before. There were no further signs of convulsive hypoglycemia in these animals and the blood glucose rose to the final hyperglycemic state. The blood glucose of dog 135 was lower at 24 hours after alloxan than at 6 or 8 hours following the treatment, but convulsive levels were not observed. Dog 129 was convulsive at 8 hours and required 160 cc. of 10 per cent glucose. The difference in response of the vagotomized-sympathectomized dogs and of intact dogs lay in the marked delay in the appearance of the hypoglycemic state in four of five

animals. The observed delay was approximately sixteen hours although it may have begun before that time; or it can be stated as requiring 2 to 3 times as long for the development of the hypoglycemic phase as was required in intact dogs injected with alloxan.

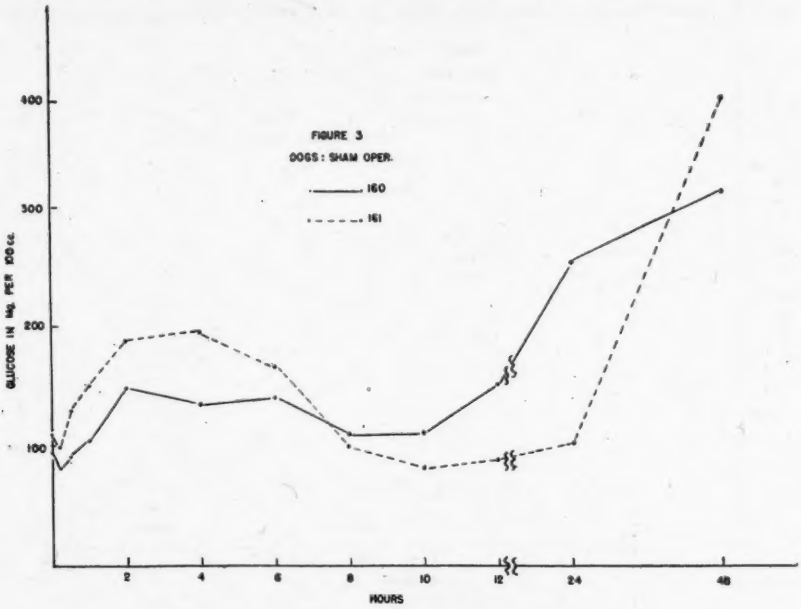
Following this it was of interest to find the effect of resection of the sympathetic chains on the response to alloxan injection. Four dogs, 213, 219, 179, and 206, were sympathectomized bilaterally in



the thoracic region with removal of approximately six ganglia and the connecting fibers. The operations were performed in two stages, allowing time for surgical recovery and healing after each operation. These dogs then were given intravenous doses of alloxan (75 mg./kg. of body weight) following the same procedure as was used with the other groups of dogs. The blood glucose values will be found in table 1 and figure 5. It can be seen that early lowering of blood glucose occurred in these dogs, and was followed by early temporary hyperglycemia, with a second decrease in blood sugar at 8 to 12 hours in dog 213 and at 8 to 24 hours in dog 219. The low glucose value at 24 hours for dog 219 is questionable for technical reasons. The dog obvously was suffering from toxic effects of the alloxan and at 29 hours the blood glucose value was found to be 564 mg./cc. The dog was hypoxic, its circulation was poor, and it was anuric; death of the animal occurred the third day. The lowest blood glucose values observed for dogs 179 and

206 occurred at 10 hours post-injection, and the usual development of permanent hyperglycemia followed.

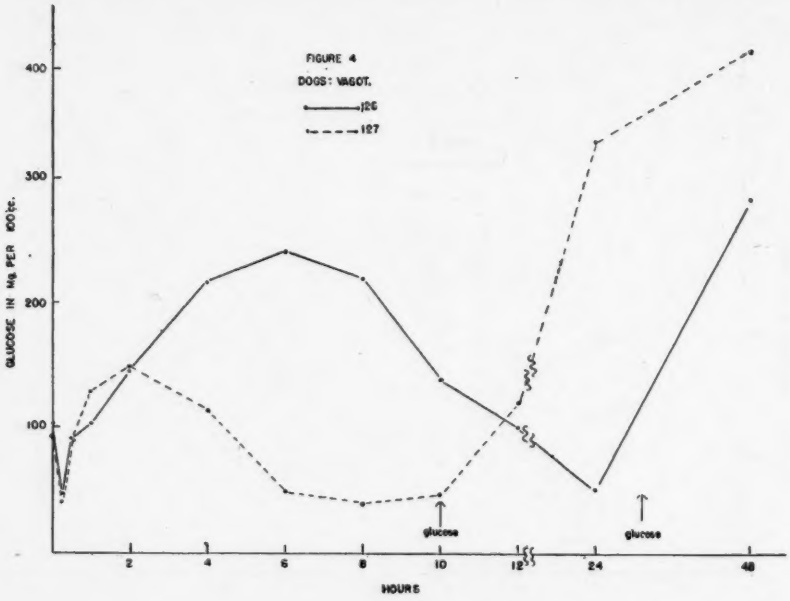
The vagi were resected in another group of 4 dogs. They were injected with alloxan in doses equivalent to those given to the dogs of the other groups. Bilateral vagotomy was performed in a single operation, using a supra-diaphragmatic approach and a single-stage resection of both vagi. After recovery from the surgical procedure the alloxan injections were given to the four dogs in the same manner as



to the dogs of the other groups. All the dogs of this group, nos. 126, 127, 139 and 166, gave decreased blood glucose values at 0.25 and 0.5 hour after the injection and 10 per cent glucose then was given intraperitoneally. Dogs 126 and 166 developed moderate early hyperglycemia followed by delayed hypoglycemic convulsions observed at 24 and 28 hours, respectively. Thus, in the vagotomized dogs it can be seen (table 1 and figure 4) that two animals responded with markedly delayed hypoglycemic convulsions, whereas the blood glucose changes were within the normal response for the other two vagotomized animals.

It is well known that adrenal cortical hormones can aid in maintenance of blood glucose. It is equally well established that operative trauma will produce adrenal hypertrophy. Thus, it seemed of importance to establish the part that such an adrenal hypertrophy might

have played in the delayed response of the dogs to alloxan injection. Accordingly, two dogs, 160 and 161, were subjected to two-stage sham operations in which the surgical trauma followed as closely as possible the extent and degree of that in resection of vagi and sympathetic chains, except the nerves were not cut. Post-operative care was in all respects the same as that given to the vagotomized-sympathectomized animals and comparable periods of time were allowed to elapse between the operations and alloxan injections. Neither of the

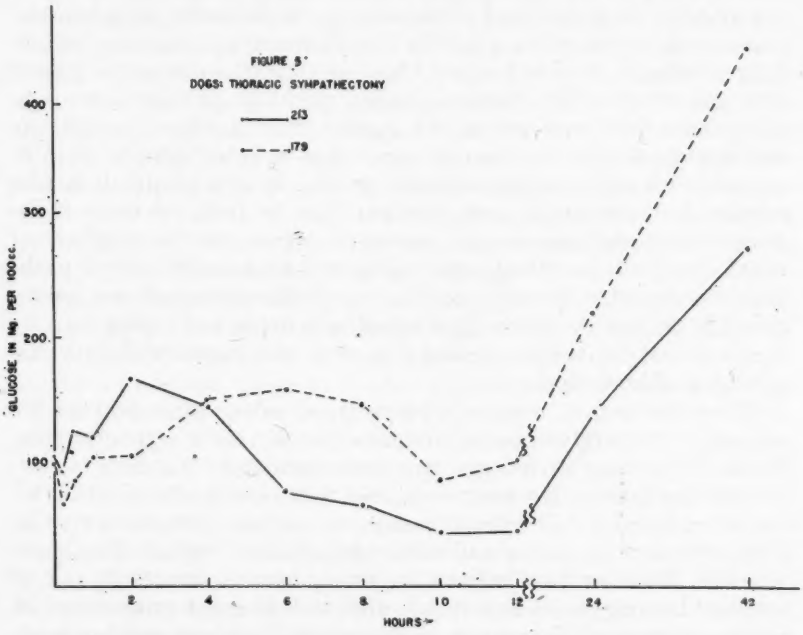


two dogs became convulsive at any stage, but the blood glucose decrease following the early hyperglycemic phase came at 8 and 10 hours in both dogs (table 1 and figure 3). The early hypoglycemic phase was not significantly low. The blood glucose changes were similar in all phases to those of intact dogs given alloxan except that very little hypoglycemia was noted. It was concluded that operative trauma had not been responsible for the delayed appearance of hypoglycemia in the experimental animals.

Since the early hypoglycemia was found in sixteen of nineteen alloxan-treated dogs, and the distribution was throughout all the groups regardless of operative procedures, we may conclude that the early hypoglycemia was not dependent upon intact innervation of the pancreas, and was probably a direct effect of the alloxan upon the pancreatic islets. The effect might possibly be upon the post-ganglionic nerve fibers in the tissue, or directly upon the beta cells of the

islets, to effect the release of insulin. The possibility of an effect upon the liver or other blood sugar raising mechanism has not been eliminated.

The secondary hypoglycemia was markedly delayed in the vagotomized-thoraco-sympathectomized dogs, and in 2 of 4 vagotomized dogs. The delay was similar to the delayed hypoglycemia noted in hypophysectomized rats given alloxan (Shipley, 1944) but on the present evidence we cannot conclude the mechanisms were the same. Kirschbaum, Wells, and Molander (1945) observed the appearance of hypoglycemic convulsions in hypophysectomized rats within 6 hrs.



after alloxan administration, however the period between hypophysectomy and alloxanization was shorter in their experiment. The dose of alloxan was larger and the fasting period was longer than for the experiment of Shipley using hypophysectomized rats. The dogs of this group that exhibited an early hypoglycemic phase did not present a marked degree of temporary hyperglycemia, hence the available glycogen may have been low at the outset.

The literature offers ample evidence that insulin secretion can be affected by nervous discharges. Etcheverry (1937) observed that hyperglycemia following glucose administration was more prolonged in vagotomized dogs than in intact ones. Hoet and Ernould (1930) using

unanesthetized rabbits, found a higher level of blood glucose and a slower return to normal in vagotomized than in normal animals.

Experiments of Zunz and La Barre (1927) using perfused dogs led to the conclusion that increased blood glucose in the circulation to the head of a dog, connected to the body only by the vagi, increased the insulin secretion from the pancreas. Removal of the cerebral hemispheres did not alter the effect, but loss of the thalamus and hypothalamus did prevent it. Feldman, Cortell, and Gellhorn (1940) found that anoxia, or metrazol, lowered blood glucose in adrenalectomized rats, but the effect was abolished by sub-diaphragmatic vagotomy. Gellhorn (1942) also pointed out that various drugs lead to a discharge over both sympatho-adrenal and vago-insulin systems and that the effects can be separated by use of adrenalectomized and vagotomized animals. On the basis of these investigations it would appear that one effect of the alloxan injection might be to stimulate a discharge over both systems; in the vagotomized animals the pathway was interrupted so that insulin release was a slower process than in animals with an intact vago-insulin system. In intact animals insulin release after alloxan treatment might thus be both by way of discharges over the vago-insulin system and from the direct effect of alloxan on the beta cells. In the vagotomized animals the nerve pathway is interrupted, leaving only the direct release from alloxan action upon the cells of the islets. This hypothesis needs to be confirmed by measurements of insulin released in normal and vagotomized animals following alloxan treatment.

Thoracic sympathectomy apparently is inadequate to prevent the temporary hyperglycemia in response to the early hypoglycemia. Further investigation, using adrenalectomized dogs, may help to clarify this mechanism. Houssay, Oria, and Sara (1945) reported that alloxan produced initial hyperglycemia even after adrenalectomy and after section of the major and minor splanchnics, but not after hepatectomy. These authors believe the liver plays an important role in terminal hypoglycemia as a result of its lack of sugar production. In the experiments of Houssay et al. dogs which had been pancreatectomized were kept under chloralose anesthesia and immediately given 100 mg. of alloxan per kg. by intravenous administration; the blood glucose values decreased during the 6 hr. observation period. Alloxan injections 24 to 48 hrs. after pancreatectomy did not produce such results. Correlation of the results they observed in recently pancreatectomized dogs with the present theory of alloxan effects is not clear. It is difficult to evaluate the results in recently operated animals under anesthesia and the exact interpretation of the data of Houssay, Orias, and Sara is not apparent in view of the surgical trauma, the prolonged use of anesthesia, and the injection of doses of alloxan as large as 100 mg. per kg. It was observed (Shipley, unpublished) that mortality was greatly increased when alloxan was given in conjunction with a bar-

biturate anesthetic, even though the dose of alloxan was lower (75 mg. per kg.) than used by Houssay et al. who also reported a very high rate of mortality in recently operated alloxan-injected dogs under chloralose.

If one accepts the anesthetized dog's response as equivalent to that of an unanesthetized animal, then one might postulate that the hypoglycemic phase after alloxan injection was a potentiation of the circulating insulin and that insulin was present in the blood and/or tissues of recently pancreatectomized dogs, but was not present 24 to 48 hrs. post-pancreatectomy.

If alloxan does potentiate the action of circulating insulin, the method of potentiation is not obvious. It has been shown (Leech and Bailey, 1945) that injected alloxan disappeared from the blood within 5 minutes after intravenous injection and (Gomori and Goldner, 1945) that portions of the pancreas, from which blood flow was obstructed during the first 8 min. after alloxan injection were unaffected by the alloxan, yet the blood glucose decreases continued for the 6 hr. observation period in the recently pancreatectomized dogs of Houssay et al. It is conceivable that the liver may play a considerable but as yet not understood role in the blood glucose changes after alloxan administration. Walpole and Innes (1946) have demonstrated that ligation of pancreatic ducts and subsequent atrophy of pancreatic acinar tissue protected the rabbit against diabetogenic doses of alloxan. They suggested that alloxan in combination with actively functioning acinar tissue was responsible for alloxan-induced necrosis of the islets and the subsequent development of diabetes, but they had no adequate explanation for the mechanism.

Sergeyeva (1943) found that vagotomy increased the percentage of α-cells in the pancreas of the cat whereas sympathectomy resulted in small decreases in percentage of α-cells. In earlier experiments Sergeyeva (1938) observed changes in secretory granules in acinar tissue after sympathetic or parasympathetic stimulation. It is conceivable that alterations in ratio of α- and β-cells, and/or alterations in the acinar tissue of the vagotomized and vagotomized-sympathectomized dogs may have played some part in the altered response of those animals to alloxan administration.

The results obtained by Covian and de Oya (1945) when alloxan (50 mg. per kg.) was administered to dogs previously treated with atropine, are of some bearing on the delayed hypoglycemic phase observed in 50% of the vagotomized and 80% of the vagotomized-sympathectomized dogs. No hypoglycemic phase was seen by Covian and de Oya in adequately atropinized dogs (0.6 to 2.0 mg. per kg.) and the final impairment of glucose tolerance was not as great as in normal dogs given alloxan.

In view of these results and those of Houssay et al. in recently pancreatectomized dogs and of Walpole and Innes in rabbits after

ligation and atrophy of the acinar tissue, the simple direct necrotic action of alloxan upon the cells of the islets appears to be inadequate explanation for the diabetogenic effect, and the blood glucose changes following alloxan administration.

From the results of Covian and de Oya and the data obtained in the present investigation, it appears that the autonomic nervous system participates in the response to alloxan administration. The precise contribution of such innervation to the mechanism of alloxan action is not as yet determined.

CONCLUSIONS

Injections of diabetogenic doses of alloxan into fasted dogs produced a tetra-phasic blood glucose change in 16 of 19 animals. The curve consisted of a brief hypoglycemia, followed by a temporary hyperglycemia, succeeded by a more prolonged second hypoglycemic phase, followed by the permanent diabetic hyperglycemia.

Vagotomized animals and vagotomized animals with supradiaphragmatic sympathectomy exhibited delayed onset of the prolonged hypoglycemic phase in 6 of 9 dogs when alloxan was administered.

Sham operation did not result in a delayed hypoglycemic phase of the blood glucose curve in response to alloxan.

Thoracic sympathectomy resulted in no significant alteration of response to alloxan. An erratic response was linked with obvious toxic effects of the alloxan.

REFERENCES

COVIAN, F. G., AND J. C. DE OYA: *Rev. clin. espan.* 17: 9. 1945.

DUNN, J. SHAW, J. KIRKPATRICK, N. G. B. MCLETCHIE AND S. V. TELFER: *J. Path. & Bact.* 55: 245. 1943.

DUNN, J. SHAW, H. L. SHEEHAN AND N. G. B. MCLETCHIE: *Lancet* 1: 484. 1943.

ETCHEVERRY, S. O.: *Compt. rend. Soc. de biol.* 126: 147. 1937.

FELDMAN, J., R. CORTELL AND E. GELLHORN: *Am. J. Physiol.* 131: 281. 1940.

GELLHORN, E.: *Autonomic Regulation.* Interscience Publishers, Inc., N. Y. 1942.

GOMORI, G., AND M. G. GOLDNER: *Proc. Soc. Exper. Biol. & Med.* 58: 232. 1945.

HOET, J., AND H. ERNOULD: *J. Physiol.* 70: 1. 1930.

HOUSSAY, B. A., O. ORIAS AND J. G. SARA: *Rev. Soc. argent. de biol.* 21(1): 30. 1945.

HUGHES, H. H., L. L. WARE AND F. G. YOUNG: *Lancet* 1: 5, 148. 1944.

KIRSCHBAUM, A., L. J. WELLS AND D. MOLANDER: *Proc. Soc. Biol. & Med.* 58: 294. 1945.

LA BARRE, J., AND VESSELOWSKY: *Arch. internat. de physiol.* 37: 188. 1933.

LEECH, R. S., AND C. G. BAILEY: *J. Biol. Chem.* 157: 325. 1945.

REINECKE, ROGER M.: *J. Biol. Chem.* 143: 351. 1942.

RIDOUT, J. H., S. W. HAM AND G. A. WRENSHALL: *Science* 100: 57. 1944.

SERGEYEVA, MARIA A.: *Anat. Rec.* 71: 319. 1938

SERGEYEVA, MARIA A.: *Rev. Can. Biol.* 2: 495. 1943.

SHIPLEY, E. G., *Thesis for Ph.D. degree,* University of Wisconsin Library, 1944.

ZUNZ, E., AND J. LA BARRE: *Compt. rend. Soc. de biol.* 96: 1400. 1927.

INHIBITION OF THYROID FUNCTION IN BEAGLE PUPPIES BY PROPYLTHIOURACIL WITHOUT DISTURBANCE OF GROWTH OR HEALTH

EDMUND MAYER

From the Chemotherapy Division, Stamford Research Laboratories
American Cyanamid Company
STAMFORD, CONNECTICUT

INTRODUCTION

ALTHOUGH dogs have been used extensively in thyroidectomy experiments, these animals have been mentioned seldom in publications on antithyroid compounds. The cabbage factor and cyanide type of antithyroids have not been tried in dogs at all, as far as we know.[1] A few papers report dogs experiments with antithyroid compounds of the sulfanilamide and thiourea types.

Danowski *et al.* (1945) administered large doses of thiouracil or thiourea to 3 adult dogs for 28 to 40 weeks without obtaining any syndrome comparable to human myxedema. Mackenzie and Mackenzie (1943) showed that sulfaguanidine altered the morphology of the thyroid glands in two puppies in the same way as in rats; the puppies were about 4 months old, and were sacrificed after 2 and 3 weeks of drug administration. In view of the low antithyroid activity of sulfaguanidine and the short period of experimentation no change in behavior or inhibition of growth was to be expected.

In our Laboratories the characteristic morphologic transformation of the thyroid has been observed in adult dogs in the course of chronic toxicity experiments with various sulfanilamides, 2-metanilamido-5-chloropyrimidine (synthesized by English *et al.*, 1946), and thiouracil. The fact that no signs of thyroid deficiency occurred in these dogs was, at that time, ascribed to the method of drug administration. On the other hand, some species like the rhesus monkey have proved to be fairly indifferent to thyroid deficiency when adult, but quite sensitive to it while growing (Fleischman *et al.*, 1943; Jailer *et al.*, 1944).

The present study was undertaken to determine whether chemical antithyroids stunt the growth of puppies as they have been shown to do in young rats (Hughes, 1944). It was decided to use two litters of beagle puppies, and to administer 6-n-propyl-2-thiouracil.[2] This com-

Received for publication January 15, 1947.

[1] Dr. Emil J. Baumann, Montefiore Hospital, confirmed this negative result of our literature search (personal communication).

[2] In the present study the compound will be termed briefly propylthiouracil.

pound, synthesized by Anderson *et al.*, (1945), has proved in rat experiments (Astwood, Bissell *et al.*, 1945) as well as in human therapy (Astwood and VanderLaan, 1945) to be more potent than any other known antithyroid compound.

In the present study experimental and control puppies will be compared mainly with respect to (1) rate of growth in terms of body weight, (2) behavior and appearance, (3) morphologic condition of the thyroid glands, (4) relative weight of internal organs, (5) ossification, and (6) maturation of gonads in the males.

The results will be discussed in the light of data on complete thyroidectomy in puppies as found in the literature.

MATERIALS AND METHODS

Two series (A and B) of beagle puppies, totaling 12 animals, were used. They came from a stock of large-sized beagles bred and raised at the Stamford Laboratories for several generations. The members of each series were litter-mates.

From the age of about 2 months the 8 males of both series were kept together in one pen, and the 4 females in another pen. Each pen consisted of an outdoor-run of about 100 square feet and an indoor-room of about 60 square feet. Soon after weaning the puppies received rations similar to those for the adult beagles which is 1 part raw horse meat+1 part Purina Dog Meal, totaling from 1 to 1½ pounds of food per dog per day.[3]

Technique of Sacrificing. An intravenous injection of nembutal was given first. As soon as the dog was anesthetized, a lethal dose of chloroform was injected into the same vein.

Post-mortem Procedures. The thyroid glands were taken out first and were weighted after removal of fatty and connective tissue. Then, from each thyroid two adjacent transverse slices, about 3 mm. thick, were removed; one slice of each gland was placed in 10% formalin (pH about 4.2) and the other in Bouin's solution (pH 1.4). The use of these two fixatives facilititates the comparison with the various microscopic pictures of dog thyroids which are found in the literature; Bouin and Zenker fixation give similar results. Erroneous interpretations of the state of the thyroid at the time of removal can and have been caused by insufficient consideration of fixation effects (details of these technical problems will be published elsewhere).

In the male dogs the testes were removed immediately after the thyroids so that samples of the testes could be fixed (in Bouin) within 15 minutes after death.

Otherwise the dissection followed the usual sequence of a complete autopsy: abdominal and thoracic situs; abdominal and thoracic organs; cranial cavity, brain and hypophysis; bones and bone marrow. Besides the thyroid glands the following organs of every dog were weighed: brain, heart, kidneys, liver, and spleen; the spleen was caused to contract in cold water before being weighed. The adrenals were weighed in all but two dogs. The

[3] Since the results of endocrinologic studies can be influenced by many auxiliary factors, additional data (concerning the history of the parents of the puppies, the seasons of their breeding, the inoculations which they received, etc.) will be supplied on request.

content and inner surfaces of stomach and intestines were inspected. The cut surfaces of the kidneys, the renal pelves and the urinary bladder were explored for possible precipitates. In each dog the femoral marrow was exposed longitudinally so that the distribution of red and yellow marrow could be observed. In 3 experimental and 3 control dogs the proximal epiphyseal junction of the tibia was examined by splitting the bone frontally.

Besides thyroid and testes the hypophyses and samples of the kidneys from each dog were fixed in Bouin, and also samples of the pancreas and thymus from one control and one experimental dog. In some experimental and control dogs the junctions of osseous rib and costal cartilage were examined in undecalcified frozen sections and in decalcified celloidin sections. All other tissues were embedded in paraffin. The sections were stained with hematoxylin-eosin and with the azan triple stain.

EXPERIMENTAL

Hughes (1944) had found that young rats were dwarfed by receiving 0.1% thiouracil in their drinking water. This dose is, on an

TABLE 1. DISTRIBUTION OF 12 PUPPIES IN EXPERIMENTAL AND CONTROL GROUPS, WITH SOME CHARACTERISTIC DATA OF BODY WEIGHTS

Series, group, sex	Dog no.	Drug administration			Body weight in kg.			Age at sacrifice
		In utero	In mother's milk	By hand	At age of 1 week	At start of propyl. admin.[a]	At age of 6 months	
Series A								
Control male	M 40	thiour.[b]	—	—	0.5	1.6	10.0	6.5 mos.
Control male	M 41	thiour.[b]	—	—	0.5	1.4	9.6	8.0 mos.
Control female	M 19	thiour.[b]	—	—	0.5	1.8	10.7	8.5 mos.
Exper. male	M 42	thiour.[b]	—	propyl.[a]	0.6	1.8	9.5	6.5 mos.
Exper. male	M 43	thiour.[b]	—	propyl.[a]	0.5	1.6	9.5	8.0 mos.
Exper. female	M 28	thiour.[b]	—	propyl.[a]	0.6	1.9	10.4	8.5 mos.
Series B								
Control male	M 48	—	—	—	0.6	2.8	12.8	6.0 mos.
Control male	M 47	—	—	—	0.5	2.6	11.8	7.5 mos.
Control female	M 50	—	—	—	0.3	2.0	9.3	8.0 mos.
Exper. male	M 46	—	—	propyl.[a]	0.5	2.7	12.4	6.0 mos.
Exper. male	M 49	—	—	propyl.[a]	0.7	2.9	13.5	7.5 mos.
Exper. female	M 51	—	—	propyl.[a]	0.5	2.2	10.0	8.0 mos.

[a] Propylthiouracil administration to the puppies started in Series A at 33 days and in Series B at 40 days of age; the dose was 30 mg./kg. body weight/day, on seven days of each week.
[b] Thiouracil given to the mother at a dose of 8.6 mg./kg. body weight/day, on six days of each week.

average, equivalent to a daily intake of 140 mg. thiouracil per kg. body weight (Astwood, 1943). As the activity of propylthiouracil in rats has proved to be at least 5 times that of thiouracil (Astwood, Bissell *et al.*, 1945), it was decided to give each experimental puppy a daily oral dose of 30 mg. propylthiouracil per kg. body weight.

A water suspension of the drug was administered orally with the use of an eye-dropper, from the 33rd day (2 weeks before completion of weaning) to the 67th day of age in Series A, and from the 40th day (last day of weaning) to the 60th day in Series B. During these periods the puppies were given their daily dose in 2 portions, at 10:00 A.M. and 3:00 P.M., from Monday to Friday, and the whole dose at once on Saturdays and Sundays of each week. From the 68th day in Series

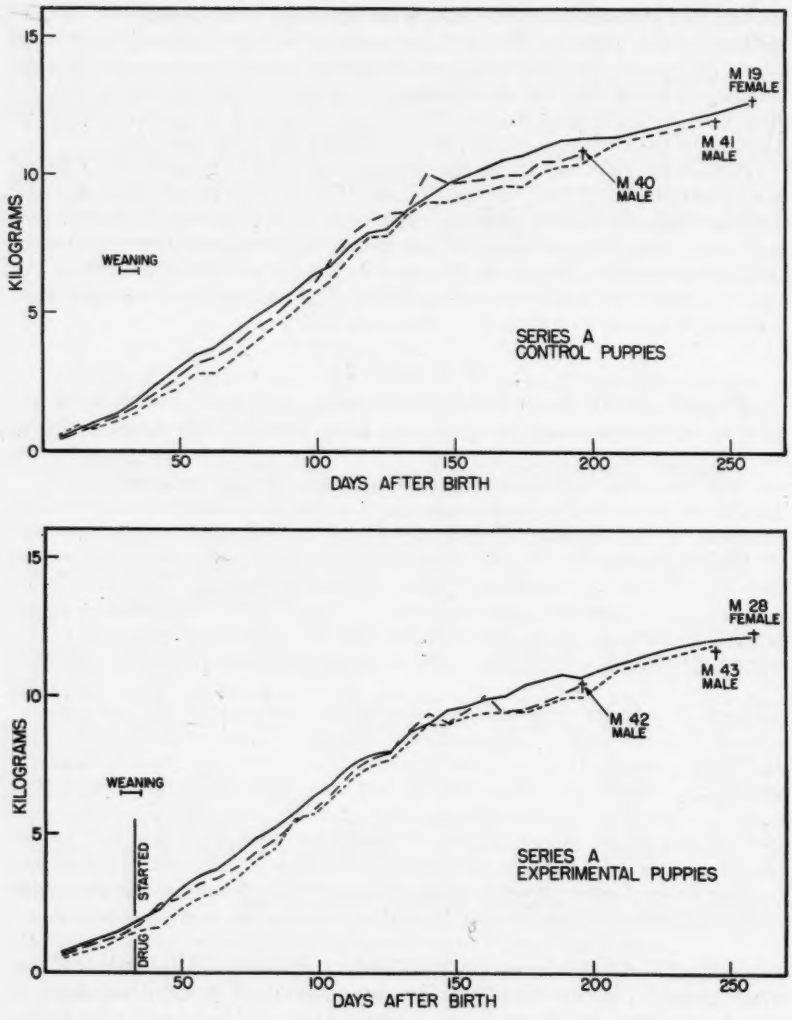


FIG. 1

A and the 61st day in Series B, until the end of the experiments, the drug was administered in gelatin capsules, once a day on every day of the week, about one hour before feeding. The actual swallowing of the capsules was checked each time.

In order to maintain the dosage, the quantities of drug were adjusted weekly to the changes of body weight. The averages of drug actually received per kg. body weight per day varied from 23–30 mg. in Series A, and from 27–30 mg. in Series B. Probably no individual

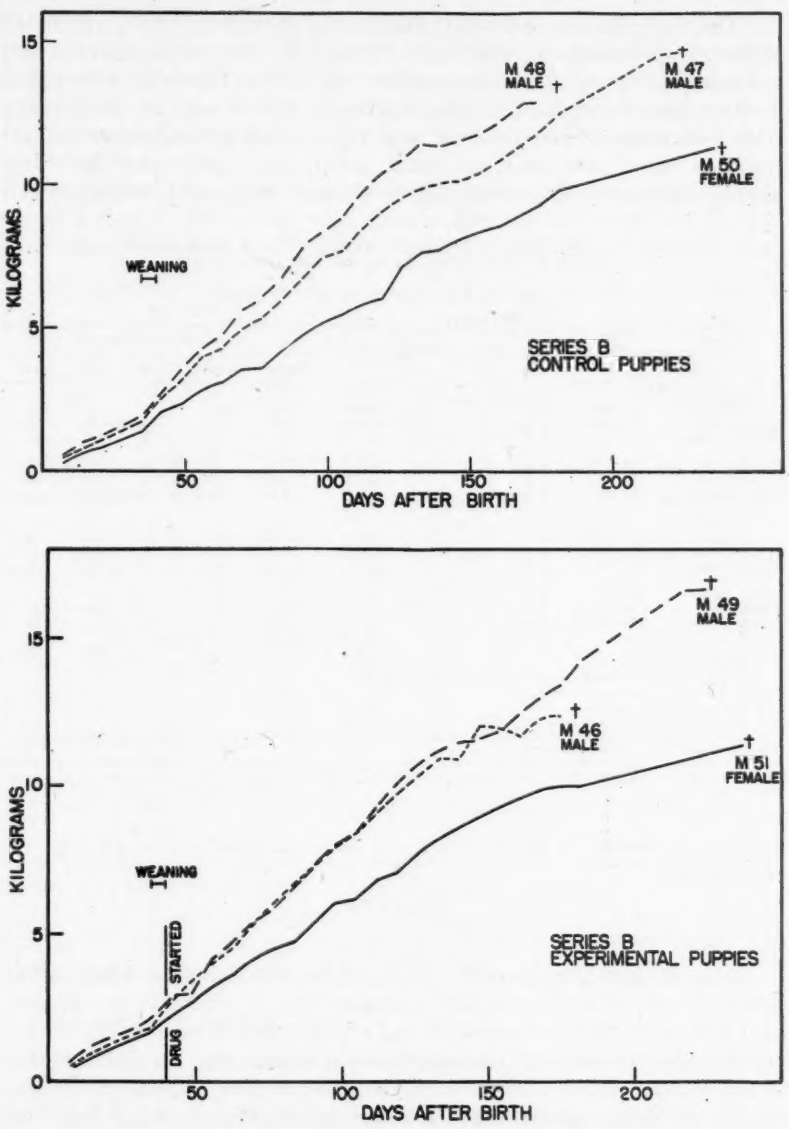


FIG. 2

received less than 21 mg. or more than 33 mg. per kg. body weight per day.

The puppies were distributed in experimental and control groups on the basis of the body weight attained before the start of drug administration (table 1).

The experiments were terminated by sacrificing the puppies at different ages, varying from 6 to 8½ months. One experimental and one control puppy of the same series (=litter), sex and age were killed and autopsied together, i.e., on the same day or on two subsequent days (cf. table 1, last column, and figs. 1 and 2). All experimental puppies were sacrificed at about 9:00 A.M.; consequently, their thyroid glands (being removed first) were inspected, weighed and

TABLE 2. HEMATOLOGIC DATA OF PUPPIES AT ABOUT 6 MONTHS OF AGE (SERIES B—6 MONTHS, SERIES A—6½ MONTHS OLD)

Series, group, sex	Dog no.	Red corpuscles		Hemoglob.[c]	White cells					
		Total[a]	Normobl.[b]		Total[d]	Differential count[e]				
						Lymph.	Segm.[f]	Trans.[g]	Eos.	Mono.
Series A										
Control male	M 40	8.0	0	8.0	14.5	55	40	2	2	1
Control male	M 41	9.2	0	8.2	16.0	55	38	4	2	1
Control female	N 19	8.7	0	8.9	21.7	45	41	8	6	0
Exper. male	M 42	9.1	0.5	8.4	18.5	47	35	10	7	1
Exper. male	M 43	7.2	0	7.0	12.5	56	35	5	2	2
Exper. female	M 28	9.0	0	9.7	14.3	71	25	2	0	2
Series B										
Control male	M 48	8.5	0	9.7	22.2	37	42	12	6	3
Control male	M 47	8.5	1	9.1	21.9	42	43	10	4	1
Control female	M 50	7.9	0	8.5	22.3	46	42	6	6	0
Exper. male	M 46	7.3	0	8.6	22.2	47	41	6	5	1
Exper. male	M 49	7.4	0	8.0	20.7	63	27	5	3	2
Exper. female	M 51	8.6	1	9.7	16.9	53	33	12	2	0

[a] Millions per mm.³
[b] As percent of 200 nucleated (white+red) cells counted.
[c] In millimols O_2 capacity per liter.
[d] Thousands per mm.³
[e] As percent of 100 white cells counted.
[f] Cells with distinct segments of their nuclei, and with or without delicate ("neutrophil") granules in otherwise unstained or very pale cytoplasm.
[g] "Transitional" cells with rod-shaped or segmented nuclei, and with granules of medium caliber in purplish stained cytoplasm (Wright stain), resembling cells listed under (f) on the one hand, and monocytes on the other hand.

preserved about 18 hours after the last administration of propylthiouracil.

RESULTS

a. *Observation during Life.*

Body Weight. The growth curves of the 6 puppies of Series A resembled each other to a surprising degree (fig. 1); there was no significant difference between controls and experimentals, or between males and females. In Series B the results were similar (fig. 2). The 2 males of the control group showed a slight difference of growth while the curves of the 2 experimental males were practically identical; the differences between the growth of the control and experimental males were not significant. While both the control and experimental females of Series B remained smaller than the males their growth curves closely resembled each other.

Appearance and Behavior. Neither the stature and proportions of the puppies nor the muscles of body and limbs showed any differences

between controls and experimentals. The hair of all control and experimental dogs was in a good condition throughout. The conjunctiva of the eyes and the mucous membranes of lips and gums were pink in all puppies at every inspection. The general behavior of control and experimental puppies was similar. All of them were lively and playful.

Hematology. A complete hematologic examination was made at the age of 6 months (Series B) or 6½ months (Series A). As table 2 shows, 2 experimental puppies (M 28 and M 49) had relatively high lympho-

TABLE 3. RELATIVE WEIGHTS OF ORGANS

Series group, sex	Dog no.	Relative weights (g. organ per kg. body weight)						
		Thyroid[a]	Adrenals[a]	Brain	Heart	Kidneys[a]	Liver	Spleen[b]
Series A								
Control male	M 40	0.06		7	7	6	37	4
Control male	M 41	0.08	0.08	6	8	5	34	3
Control female	M 19	0.08	0.10	6	7	5	35	4
Exper. male	M 42	0.22		7	8	5	33	3
Exper. male	M 43	0.19	0.10	7	8	6	36	4
Exper. female	M 28	0.20	0.10	6	9	6	36	6
Series B								
Control male	M 48	0.10	0.08	7	8	8	49	4
Control male	M 47	0.09	0.07	5	7	6	30	3
Control female	M 50	0.08	0.08	7	7	6	40	4
Exper. male	M 46	0.31	0.09	7	9	8	43	7
Exper. male	M 49	0.33	0.08	5	8	7	33	3
Exper. female	M 51	0.41	0.09	7	9	7	45	5

[a] Sum of left and right organ.
[b] Spleens were completely contracted when weighed except for that of M46

cyte counts, exceeding 60 %. Otherwise there were no significant differences between control and experimental puppies.

Diseases. There were no signs of disease in any of the 12 puppies during the whole period of observation except for a slight intestinal disturbance in the control puppy M 50 at the age of 8 months.

b. Post-mortem Findings.

Fat. The experimental and control puppies had a similar amount of subcutaneous and intra-abdominal fat.

Thyroid Glands. The thyroids of the 6 controls were small and pink, while those of the 6 experimental puppies were large and purple, frequently showing conspicuously congested veins. The relative weights are given in table 3. Microscopically, the thyroids of the 6 control dogs showed the typical storage phase as illustrated in figs. 3, 4, 7 and 8. The picture was quite homogeneous in all samples examined except for some formalin-fixed sections of the thyroid of M 46;

these sections contained "microfollicular" admixtures, i.e., very small (fetal or atrophic) follicles with hardly any lumen. The thyroids of the experimental dogs showed the characteristic picture of "morphologic activation" as illustrated in figs. 5, 6, 9 and 10. This microscopic picture was very pronounced in 5 of the experimental dogs, but less pronounced in the thyroid of M 51, in so far as its follicles had fairly regular outlines and were lined by an epithelium of moderate height (this thyroid had the highest relative weight, cf. table 3).

The triple azan stain (in Bouin-fixed sections) confirmed the impression gained with hematoxylin eosin stain that diluted colloid was predominant in the experimental thyroids and concentrated colloid in the control glands. In all experimental thyroids, except M 51, the great majority of follicles contained colloid which was unstained (gray) or of a very pale color. In the control glands every follicle contained well-stained colloid, mostly red, pink or purple, rarely blue.

Parathyroid Glands (inside the thyroid capsule). In the experimental puppies the parathyroids, before fixation, were usually paler than the surrounding thyroid tissue. With regard to size, no differences were observed between controls and experimentals. Large parts of the parathyroids were present in the histological sections of 5 control and 4 experimental thyroids. All of them showed the same type of small cuboidal cells, arranged in garlands. Follicle-like structures were equally scarce in controls and experimentals. The number of open blood vessels appeared, usually, increased in the experimentals.

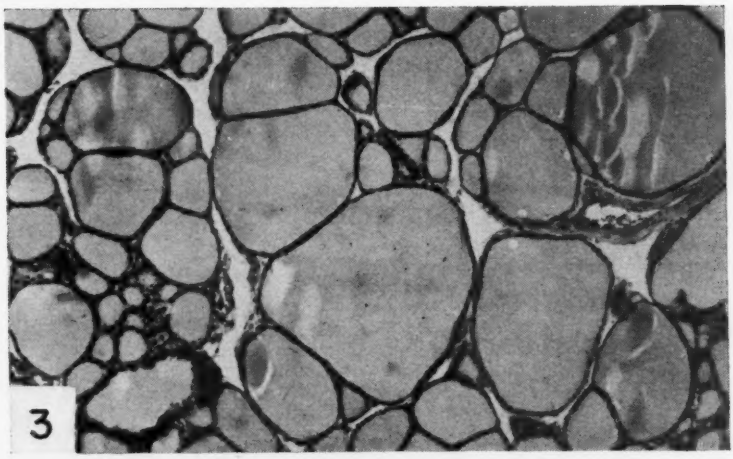
Hypophysis. There were no conspicuous differences in the sizes of hypophyses of control and experimental dogs. Microscopically, no attempt was made to determine the proportions of the various cell types in the anterior lobe. Nothing pathological was seen.

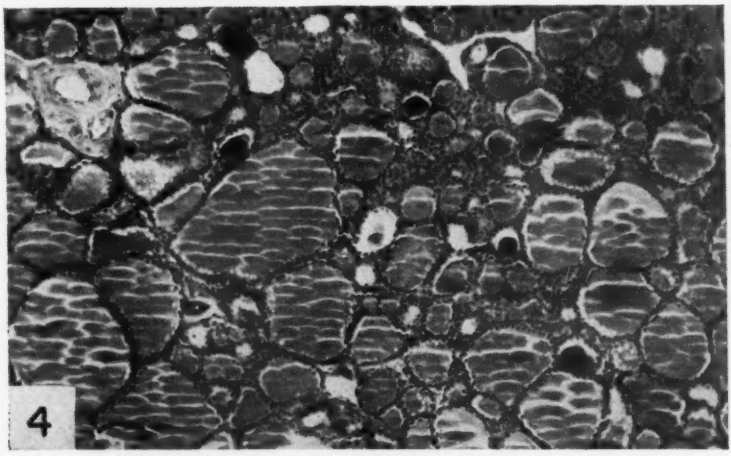
Adrenals. It appears from the relative weights in table 3 that there were no significant differences between the glands of control and of experimental dogs. Cross sections through the fresh organs showed the pale yellow cortex and the whitish-gray medulla in their proper proportions, both in the 6 controls and the 6 experimental dogs. No microscopic examinations were made.

Pancreatic Islets. The pancreases of control M 48 and of experimental M 46 were examined microscopically with routine methods and no differences observed. No attempt was made to differentiate between the various cell types in the islets.

Thymus. Microscopical examinations were made in control M 48 and experimental M 46; cortex, medulla, and the tiny Hassal's bodies were similar in the 2 dogs.

Testes. No conspicuous differences in position, size, or appearance of the cut surface were observed between the testes of controls and those of experimental dogs. Microscopically the following conditions were found. At 6 and 6½ months the convoluted seminiferous tubules



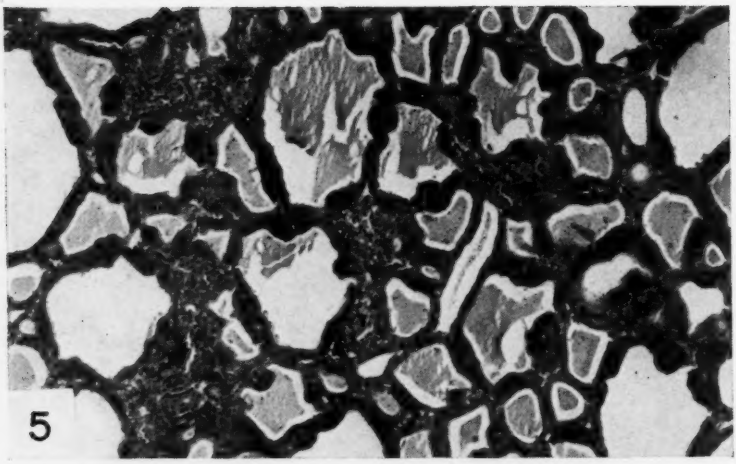


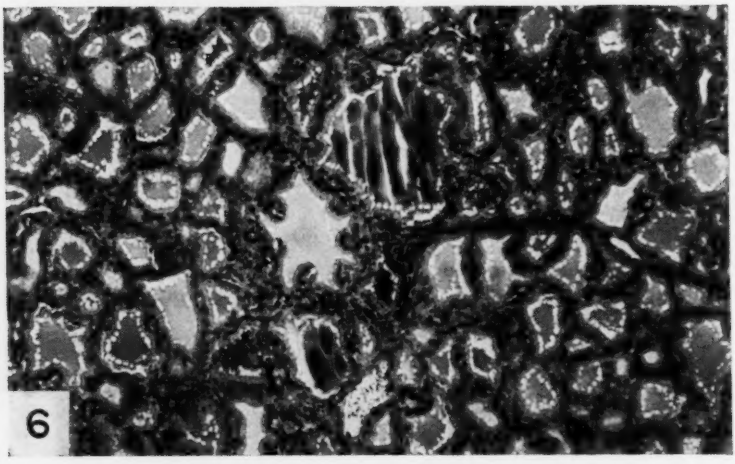
FIGS. 3 and 4. Control Dog M 48. Thyroid in storage phase; paraffin sections about 7 micra thick, hematoxylin and eosin stain. Magnification 100×.

FIG. 3, formalin fixation: smooth outlines of follicles; no shrinkage of colloid; shallow epithelium (cf. higher magnification on Fig. 7).

FIG. 4, Bouin fixation: follicles smaller than in Fig. 3, but with equally smooth outlines; colloid with marginal vacuoles; cuboidal epithelium (cf. higher magnification on Fig. 8).

of the experimental dogs showed no mature spermatozoa and frequently not more than 2 layers of spermatogenetic cells. The 6 months control had 2 layers of spermatogenetic cells in most tubules, but a few more advanced tubules contained a very small number of mature spermatozoa. The 6½ months' control had 3 or more layers of spermatogenetic cells and a sizable number of mature spermatozoa. At 7½ and 8





FIGS. 5 and 6. Experimental Dog M 46. Thyroid showing pronounced morphologic activation; paraffin sections about 7 micra thick, hematoxylin and eosin stain. Magnification 100×.

FIG. 5, formalin fixation: irregular outlines of follicles; colloid split into shreds and lost from many follicles; high epithelium (cf. higher magnification on Fig. 9).

FIG. 6, Bouin fixation: follicles smaller than in Fig. 5, some of them with stellate outlines; colloid with numerous marginal vacuoles, but still coherent and present in every follicle; high epithelium (cf. higher magnification on Fig. 10).

months the tubules contained 3 or more layers of cells and large masses of mature spermatozoa, both in the controls and in the experimental dogs. Possibly, there was a slight retardation of maturation of testes in the experimental dogs which was compensated after the 7th month.

Ovaries. The ovaries of control and experimental dogs appeared equally immature, as they showed neither scars on the surface nor corpora lutea.

Skeletal System. (a) Tibia, frontal section through proximal epiphysis. In the 6 months' puppies (control M 48 and experimental M 46), as well as in the 6½ months puppies (control M 40 and experimental M 42) the epiphyseal lines were about 1 mm. thick, of bluish (cartilage) color, with regular borders. In the 8 months' old puppies (control M 50 and experimental M 51) the line was only a fraction of a millimeter thick, and more whitish than blue. (b) Ribs. The junction of bone and costal cartilage showed equally regular border lines in controls (M 48 and M 40) and experimentals (M 46 and M42). Microscopically, there was no difference of ossification between experimentals and controls: the columns of enlarged cartilage cells were of equal length, and in the trabeculae of the spongy bone near the cartilage there was a similar distribution of calcium (staining purple with hematoxylin in undecalcified sections).

Femoral Marrow. Various proportions of red and yellow marrow were found in the experimentals as well as in the controls; completely red marrow was present in one control (M 41) and one experimental (M 46). These are normal variations in dogs at the end of their growing period. Evidently there was no correlation between the drug administration and the activity of the bone marrow.

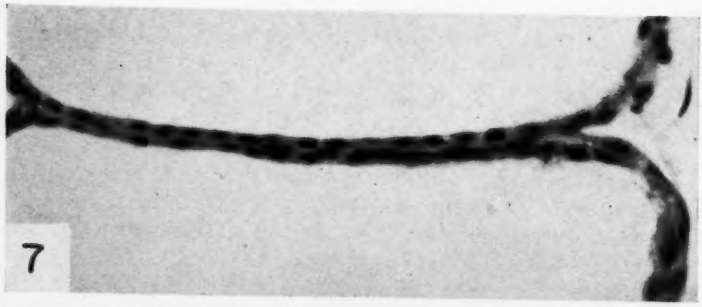
Other Organs. Nothing of significance with relation to the present study was found in the other organs. The only pathological condition seen were fresh hemorrhages in the lungs and under the epicardium of all dogs, and in some subcapsular thyroid follicles of one dog (M 46). These are evidently results of the intravenous chloroform injections.

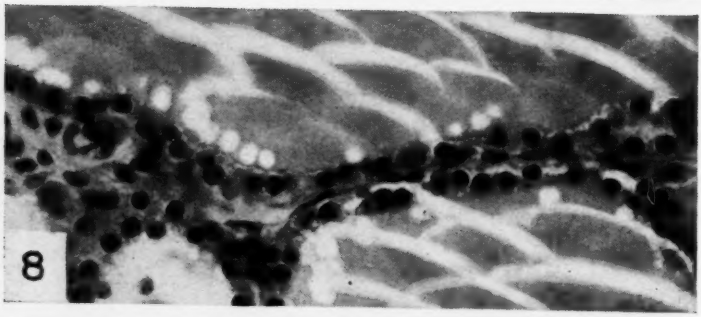
DISCUSSION

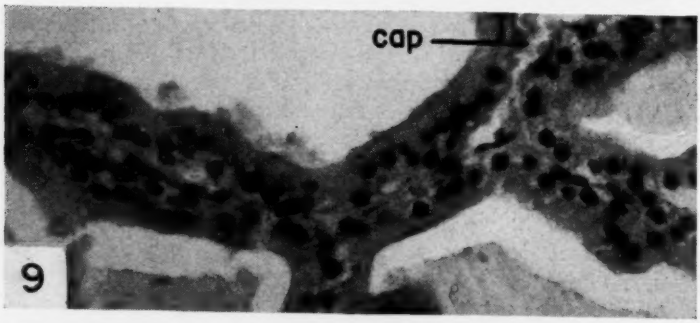
A comparison between the experimental and control puppies showed no differences with respect to their rate of growth, general appearance and behavior, amount of subcutaneous and intraperitoneal fat, relative weight of inner organs, or ossifisation of tibia and ribs. There was one significant difference only, namely, the condition of the thyroid glands. In all experimental puppies the thyroids were greatly enlarged; their average relative weight was 3.5 times that of the control glands. All of the experimental glands had an increased vascularization. Their histologic picture was that of pronounced epithelial hyperplasia, with decreased concentration of colloid.[4]

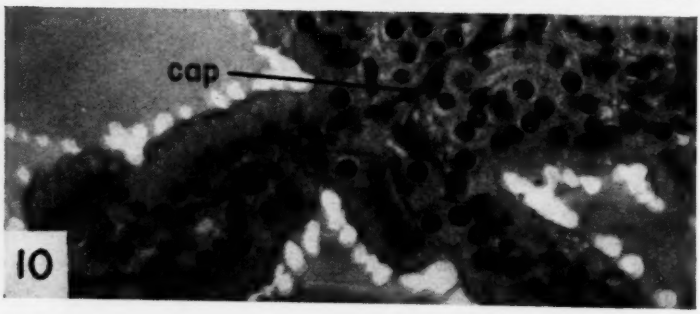
Under the conditions of the present study these alterations in the thyroids of experimental animals can only be interpreted by analogy to the 3-phase mechanism which has been demonstrated in rats: (1) drug induced inhibition of thyroxin production, (2) increased forma-

[4] Evidences that most variations of colloid are variations in protein concentration will be published elsewhere.


7


8


cap
9


cap
10

tion of thyrotropic hormone in the pituitary as a response to the thyroxin deficiency in the circulating blood, and (3) morphologic activation of the thyroid as a response to the increased stimulation by thyrotropic hormone (Mackenzie and Mackenzie, 1943; Astwood, Sullivan *et al.*, 1943).

There is little doubt that, in this mechanism, the morphologic activation of the thyroid is futile in so far as it does not overcome the functional inactivation. It is difficult to tell whether or not a small fraction of functional activity is preserved in such glands. In any case the inhibition of thyroid function appears to be of no consequence with regard to the growth, development and health of the puppies.

These results are somewhat paradoxical. Evidently, the thyroid function is not needed in growing beagles under ordinary conditions, yet the anterior pituitary reacts to the thyroxin deficiency as if there were an urgent demand for this hormone.

On the other hand, there is a good agreement between the results of propylthiouracil administration and the results of thyroidectomy in growing dogs. A careful evaluation of the literature shows that, as a rule, complete thyroidectomy does not produce dwarfism in puppies.

Dye and Maughan (1929) used 1 litter of 5 and another litter of 4 dogs. In 3 puppies of the 1st litter and 2 of the 2nd litter the thyroids were removed completely, at 5 and 6 weeks of age respectively. Only 1 of the 5 operated puppies remained conspicuously small. The rate of growth (body weight) of the 4 others did not differ significantly from that of the control litter-mates.[5] Binswanger (1936) made complete thyroidectomies in 5 puppies which came from 5 different litters, and compared the development of each operated puppy with that of a control litter-mate of similar weight, and usually of the same sex. Three of the puppies were operated between $2\frac{1}{2}$ and 4 weeks of age, 1 at 7 weeks and 1 at 4 months. None of the operated puppies were

[5] These facts appear much clearer from the curves of growth, figs. 1 and 2, of Dye and Maughan than from the calculations on their table 1.

FIGS. 7, 8, 9, and 10. Higher magnifications (500×) of parts from the same thyroid sections which are shown at lower power in figs. 3, 4, 5, and 6.

FIG. 7. Control Dog M 48. Storage phase, formalin fixation. Epithelium shallow; nuclei oval, very dark, without visible structure; (for colloid see fig. 3).

FIG. 8, same thyroid as in fig. 7. Storage phase, Bouin fixation. Epithelium cuboidal; spherical nuclei, with distinct structure, located in the center of each cell; colloid with marginal vacuoles.

FIG. 9, Experimental Dog M 46. Morphologic activation, formalin fixation. Epithelium high; spherical nuclei, very dark, located in basal part of cells; capillaries (cap) remained open with this fixative; (for colloid see fig. 5).

FIG. 10, same thyroid as in fig. 9. Morphologic activation, Bouin fixation. Epithelium still higher than in fig. 9, and with more distinct cell boundaries; spherical nuclei, with distinct structure, situated very close to cell base; capillaries (cap) compressed by the fixative; colloid with marginal vacuoles.

dwarfed. There were some temporary depressions of the growth curves after operation, but at sacrifice, several months after the thyroidectomy, only 1 puppy weighed less (about 15%) than its control, while the body weights of the 4 other operated puppies exceeded those of their respective controls by 4 to 25%. Two partially thyroidectomized puppies from 2 different litters gave opposite results: at sacrifice one of them weighed 47% less than its control, and the other one 52% more than its control. Binswanger rightly interprets these variations as caused by genetic differences in his mongrel dogs. Binswanger thinks that neither his results nor those of Dye and Maughan agree with the statements and pictures in Biedl's (1913) well-known book. However, there is a close resemblance between the dwarf puppy of Dye and Maughan and one of those pictured by Biedl. Apparently, some dogs cannot grow properly after thyroidectomy. There is no doubt that in this type of investigation the results obtained with mongrels will be less definite than those obtained with dogs of the same race such as the beagles used in the present study.

Some authors seem to take it for granted that thyroidectomy interferes with the growth of puppies. For instance, Trendelenburg's (1934) review gives the impression that, after thyroidectomy, dogs are dwarfed quite regularly ("ausserordentlich starke Hemmung des Skeletwachstums"). However, in appraising experiments of this type 4 factors have to be considered: the natural variability of growth-rate in mongrel litter-mates, their variable reactivity to thyroid deficiency, the temporary disturbance caused by the operation, and, finally, the inevitable reduction in number of parathyroids or injury to those remaining.

It may be useful to discuss the possible role of the parathyroid in the growth of puppies on the basis of data contained in the papers of Dye and Maughan and of Binswanger.

Binswanger described slightly abnormal proportions of long bones and a temporary retardation of ossification in the thyroidectomized puppies. His observations of the proportions of bones agree, more or less, with those of Dye and Maughan. Dye and Maughan stated that no tetany occurred in their thyroidectomized puppies. Two of Binswanger's puppies died from delayed tetany several months after operation,[6] and in these none of the parathyroids which had been left in situ at operation was found at autopsy; in 2 other puppies only 1 of the 2 parathyroids which had been left in situ was still present at autopsy.

In the light of these observations, and in view of the fact that the thyroid as well as the parathyroid glands play a role in calcium metabolism, it seems probable that the skeletal conditions described by Dye and Maughan and by Binswanger have been caused, at least in part, by parathyroid deficiency.

[6] On delayed and latent tetany in adult dogs cf. Marine (1914).

As Marine (1914) stated about 30 years ago, it is exceedingly difficult in all carnivora to remove the thyroid completely without producing a transient or permanent parathyroid deficiency. One may say now that a potent antithyroid drug like propylthiouracil is a suitable tool for eliminating the thyroid function without damaging the parathyroids. When, in the present study, the interior parathyroid glands were examined morphologically, no differences were found between those of the experimental puppies and those of the controls, except for a higher degree of vascularization ("hyperemia") in the experimentals.

Another advantage of an antithyroid drug over thyroidectomy is the fact that it will inactivate scattered thyroid tissue, particularly frequent in dogs.

The usefulness of antithyroid drugs in such experiments will, of course, increase when the blood drug level and the thyroxin production can be determined. With respect to the present study, both the blood drug level and the thyroxin production can only be discussed in an indirect and approximate way.

On the one hand, there is a good deal of evidence that the drug level in the blood decreases rapidly (Christensen, 1946, footnote 3; R. H. Hall, unpublished data). Therefore there is the possibility that the single daily doses administered to the puppies did not maintain the necessary blood levels. On the other hand, it has been shown by R. F. Goddard at our Laboratories that single daily doses of propylthiouracil inhibited the growth in young rats which had received the drug from their 11th to their 32nd day of age. These results were obtained by subcutaneous injections of 10 mg. drug per kg. body weight per day. In view of this fact the failure to inhibit the growth of puppies with 3 times the rat dose of propylthiouracil can hardly be attributed to the technique of administration. Rats are dwarfed by early thyroidectomy as well as by antithyroid drugs. Puppies are, as a rule, not dwarfed by thyroidectomy. One would not, therefore, expect them to be dwarfed by inactivation of the thyroid through propylthiouracil or similar drugs.

These facts indicate indirectly that dogs do not need the function of the thyroid under normal conditions. A direct approach to this question has been made by Danowski *et al.* (1945) who tried to determine the concentration of diiodotyrosine and thyroxine in the circulating blood of normal dogs. They found the values to be so low that, in some cases, no organically bound iodine could be detected with the method used. (It would be interesting to ascertain whether higher concentrations of diiodotyrosine and thyroxin occur in the blood of dogs after long exposure to low environmental temperature.)

It is difficult to find in the literature physiological data which could shed more light on the role of the thyroid gland in dogs. Several authors have determined blood cholesterol before and after complete

dwarfed. There were some temporary depressions of the growth curves after operation, but at sacrifice, several months after the thyroidectomy, only 1 puppy weighed less (about 15%) than its control, while the body weights of the 4 other operated puppies exceeded those of their respective controls by 4 to 25%. Two partially thyroidectomized puppies from 2 different litters gave opposite results: at sacrifice one of them weighed 47% less than its control, and the other one 52% more than its control. Binswanger rightly interprets these variations as caused by genetic differences in his mongrel dogs. Binswanger thinks that neither his results nor those of Dye and Maughan agree with the statements and pictures in Biedl's (1913) well-known book. However, there is a close resemblance between the dwarf puppy of Dye and Maughan and one of those pictured by Biedl. Apparently, some dogs cannot grow properly after thyroidectomy. There is no doubt that in this type of investigation the results obtained with mongrels will be less definite than those obtained with dogs of the same race such as the beagles used in the present study.

Some authors seem to take it for granted that thyroidectomy interferes with the growth of puppies. For instance, Trendelenburg's (1934) review gives the impression that, after thyroidectomy, dogs are dwarfed quite regularly ("ausserordentlich starke Hemmung des Skeletwachstums"). However, in appraising experiments of this type 4 factors have to be considered: the natural variability of growth-rate in mongrel litter-mates, their variable reactivity to thyroid deficiency, the temporary disturbance caused by the operation, and, finally, the inevitable reduction in number of parathyroids or injury to those remaining.

It may be useful to discuss the possible role of the parathyroid in the growth of puppies on the basis of data contained in the papers of Dye and Maughan and of Binswanger.

Binswanger described slightly abnormal proportions of long bones and a temporary retardation of ossification in the thyroidectomized puppies. His observations of the proportions of bones agree, more or less, with those of Dye and Maughan. Dye and Maughan stated that no tetany occurred in their thyroidectomized puppies. Two of Binswanger's puppies died from delayed tetany several months after operation,[6] and in these none of the parathyroids which had been left in situ at operation was found at autopsy; in 2 other puppies only 1 of the 2 parathyroids which had been left in situ was still present at autopsy.

In the light of these observations, and in view of the fact that the thyroid as well as the parathyroid glands play a role in calcium metabolism, it seems probable that the skeletal conditions described by Dye and Maughan and by Binswanger have been caused, at least in part, by parathyroid deficiency.

[6] On delayed and latent tetany in adult dogs cf. Marine (1914).

As Marine (1914) stated about 30 years ago, it is exceedingly difficult in all carnivora to remove the thyroid completely without producing a transient or permanent parathyroid deficiency. One may say now that a potent antithyroid drug like propylthiouracil is a suitable tool for eliminating the thyroid function without damaging the parathyroids. When, in the present study, the interior parathyroid glands were examined morphologically, no differences were found between those of the experimental puppies and those of the controls, except for a higher degree of vascularization ("hyperemia") in the experimentals.

Another advantage of an antithyroid drug over thyroidectomy is the fact that it will inactivate scattered thyroid tissue, particularly frequent in dogs.

The usefulness of antithyroid drugs in such experiments will, of course, increase when the blood drug level and the thyroxin production can be determined. With respect to the present study, both the blood drug level and the thyroxin production can only be discussed in an indirect and approximate way.

On the one hand, there is a good deal of evidence that the drug level in the blood decreases rapidly (Christensen, 1946, footnote 3; R. H. Hall, unpublished data). Therefore there is the possibility that the single daily doses administered to the puppies did not maintain the necessary blood levels. On the other hand, it has been shown by R. F. Goddard at our Laboratories that single daily doses of propylthiouracil inhibited the growth in young rats which had received the drug from their 11th to their 32nd day of age. These results were obtained by subcutaneous injections of 10 mg. drug per kg. body weight per day. In view of this fact the failure to inhibit the growth of puppies with 3 times the rat dose of propylthiouracil can hardly be attributed to the technique of administration. Rats are dwarfed by early thyroidectomy as well as by antithyroid drugs. Puppies are, as a rule, not dwarfed by thyroidectomy. One would not, therefore, expect them to be dwarfed by inactivation of the thyroid through propylthiouracil or similar drugs.

These facts indicate indirectly that dogs do not need the function of the thyroid under normal conditions. A direct approach to this question has been made by Danowski *et al.* (1945) who tried to determine the concentration of diiodotyrosine and thyrosine in the circulating blood of normal dogs. They found the values to be so low that, in some cases, no organically bound iodine could be detected with the method used. (It would be interesting to ascertain whether higher concentrations of diiodotyrosine and thyroxin occur in the blood of dogs after long exposure to low environmental temperature.)

It is difficult to find in the literature physiological data which could shed more light on the role of the thyroid gland in dogs. Several authors have determined blood cholesterol before and after complete

thyroidectomy. In some animals concentrations became, and remained, high after the operation, but in others no change was observed (Thompson and Long, 1941; Chaikoff *et al.*, 1941). As the dogs used were mongrels, their different reactions may have been caused by genetic differences. In the present study no cholesterol analyses were made.

The basal metabolism cannot be determined in young restless puppies, as Binswanger (1936) emphasized. When 3 of his thyroidectomized puppies had reached a more mature phase, he determined their basal metabolism and found decreased values in 2 of them, but not in the 3rd. Probably, in the present study, the metabolic rates of the experimental puppies were similar to those of the controls, since there were no differences in their rates of growth, their stature, and the relative weights of their internal organs. Under these circumstances lower metabolic rates in the experimental puppies could occur only if decreased intake (or utilization) of food were accompanied by decreased activity in such a way that the resulting balance of growth equalled precisely that of the controls. This seems unlikely.

There is still the possibility that the growth of puppies could be inhibited by administration of propylthiouracil if, at the same time, their metabolism would be altered by other factors such as low environmental temperature, or restricted food supply, or both. Evidently, the experimental control of the 3 factors would involve a large research program, as little work of this type seems to have been done in dogs.[7]

SUMMARY

Beagle puppies of 2 litters received large daily doses of 6-n-propyl-2-thiouracil from their 5th or 6th week of age until sacrifice at $6\frac{1}{2}$ to 8 months. The experimental puppies were compared with control littermates of the same sex, both by observation during life and postmortem analysis. At autopsy the thyroids of all experimental puppies showed the pronounced morphologic activation characteristically produced by antithyroid compounds, while those of the controls were in the typical storage phase.

Although a lack of thyroid hormone production must be assumed in the experimental puppies, there was no difference between them and the controls with respect to rate of growth, general health, appearance and behavior, amount of subcutaneous and intra-abdominal fat, relative weights of internal organs, and, finally, ossification of tibial epiphysis and ribs. A retardation in the development of spermatogenesis in the experimental dogs was compensated after the 7th month of age.

[7] There is a paper by Boldyreff (1908) from Pawlow's laboratory, entitled "On the effect of high and low temperature in thyroidectomized dogs"; the author described what now would be called typical manifestations of tetany which were released by exposing the (adult) dogs to sudden changes of temperature.

The results obtained with 6-n-propyl-2-thiouracil agree with the data in the literature which show that, as a rule, complete thyroidectomy does not produce dwarfism in puppies. Minor disturbances of skeletal development, observed after thyroidectomy in puppies, may have been caused by parathyroid injury.

From these facts it can be concluded that beagles, and probably most dogs, do not need the thyroid function under ordinary conditions.

As an experimental tool, a potent antithyroid drug has some advantages over thyroidectomy in so far as the parathyroids are not damaged, and scattered thyroid tissue is reached by the drug.

Dogs of the same race, such as the beagles in the present study, should be given preference over mongrels in experiments on growth.

ACKNOWLEDGMENTS

We wish to acknowledge the assistance and advice of numerous members of the staff of the Chemotherapy Division in all phases of this study. We are indebted to the staff of the Microscopical Department of the Physics Division for generous cooperation in preparing the photomicrographs.

REFERENCES

ANDERSON, G. W., I. F. HALVERSTADT, W. H. MILLER AND R. O. ROBLIN, JR.: *J. Am. Chem. Soc.* 67: 2197. 1945.

ASTWOOD, E. B., J. SULLIVAN, A. BISSELL, AND R. TYSLOWITZ: *Endocrinology* 32: 210. 1943.

ASTWOOD, E. B.: *J. Pharmacol. & Exper. Therap.* 78: 79. 1943.

ASTWOOD, E. B., A. BISSELL AND A. M. HUGHES: *Endocrinology* 37: 456. 1945.

ASTWOOD, E. B., AND W. P. VANDERLAAN: *J. Clin. Endocrinol.* 5: 424. 1945.

BIEDL, A.: Innere Sekretion. Berlin and Vienna: Urban and Schwarzenberg. 2nd. ed. 1913.

BINSWANGER, F.: *Endokrinologie* 17: 22 and 150. 1936.

BOLDYREFF, W. N.: *Zentralbl. f. Physiol.* 22: 310. 1908.

CHAIKOFF, I. C., C. ENTENMAN, G. W. CHANGUS AND F. L. REICHERT: *Endocrinology* 28: 797. 1941.

CHRISTENSEN, H. N.: *J. Biol. Chem.* 162: 27. 1946.

DANOWSKI, T. S., E. B. MAN AND A. W. WINKLER: *Endocrinology* 38: 230. 1946.

DYE, J. A., AND G. H. MAUGHAN: *Am. J. Anat.* 44: 331. 1929.

ENGLISH, J. P., J. H. CLARK, R. G. SHEPHERD, H. W. MARSON, J. KRAPCHO AND R. O. ROBLIN, JR.: *J. Am. Chem. Soc.* 68: 1039. 1946.

FLEISCHMANN, W., H. B. SHUMAKER AND W. L. STRAUS: *Endocrinology* 32: 238. 1943.

HUGHES, A. M.: *Endocrinology* 34: 69. 1944.

JAILER, J. W., W. M. SPERRY, E. T. ENGLE AND G. SMELSER: *Endocrinology* 35: 27. 1944.

MACKENZIE, C. G., AND J. B. MACKENZIE: *Endocrinology* 32: 185. 1943.

MARINE, D.: *J. Exper. Med.* 19: 89. 1914.

THOMPSON, K. W., AND C. N. H. LONG: *Endocrinology* 28: 715. 1941.

TRENDELENBURG, P.: Die Hormone (edit. by O. Krayer). Berlin: J. Springer. 1934.

THE RELATION OF THE HYPOPHYSIS, OVARY, PLACENTA AND FETUS TO THE DEVELOPMENT OF ANEMIA DURING THE LAST HALF OF PREGNANCY IN THE RAT[1]

JUANITA S. NEWCOMER[2]
From the Department of Zoology, Cornell University
ITHACA, NEW YORK

THE NUTRITIONAL aspects of anemia of pregnancy in man and the rat have been studied extensively, but no attempt has been made to investigate this anemia in the light of the activity of certain endocrine glands. This paper deals with a study of what may be considered the essential endocrine glands involved in pregnancy of the rat; that is, the hypophysis, ovary and "placenta," and their relationship to anemia of pregnancy. The role of the fetus also will be considered in the analysis of the problem. It is realized that other endocrine glands may possibly affect the blood picture in pregnancy, but this study has been limited to the factors mentioned above. As an index to the degree of anemia, the hemoglobin concentration and erythrocyte number were determined in pregnant rats after removal of the hypophysis, ovaries, placentae and fetuses, each alone and in all possible combinations.

The effect of the various endocrine secretions on the composition of the blood in non-pregnant rats has been previously studied. Hypophysectomy results in a lowered red blood cell count, lowered hemoglobin concentration and reticulopenia (for complete bibliography, see Meyer, Thewlis and Rusch, 1940; Crafts, 1941; Vollmer and Gordon, 1941). Recent experiments dealing with the ovary in relation to the blood picture demonstrate that estrogens depress red cell formation (Steinglass, Gordon and Charipper, 1941; Vollmer and Gordon, 1941; Gordon and Charipper, 1943). Progesterone, however, has been considered very little in relation to anemia. Vollmer and Gordon (1941) have shown that in hypophysectomized rats progesterone treatment caused no significant increase in red blood cell or hemoglobin levels. It was possible to study the combined effects of hypophysectomy and pregnancy on hemoglobin and red cell levels in

Received for publication January 20, 1947.
[1] Aided by a grant from the Sage Fund provided by the Cornell University Trustee-Faculty Committee on Research administered by Dr. S. L. Leonard.
[2] Based upon a thesis submitted to the Graduate School of Cornell University in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

the rat since pregnancy continues without interruption and parturition occurs normally in rats hypophysectomized as early as the 11th day of gestation (Pencharz and Long, 1931; 1933; Selye, Collip and Thomson, 1933).

Many writers have attributed the anemia of pregnancy to dietary deficiencies. If the anemia of pregnancy in the rat were due merely to a deficiency of iron, sufficient addition of the mineral would prevent the condition, other things being equal. In the experiments of Mitchell and Miller (1931), Beard and Myers (1933) and Van Donk, Feldman and Steenbock (1934) pregnant rats became anemic in spite of adequate mineral supplements. Since the anemia of pregnancy in the rat had not been corrected by supplemented diets, it seemed that the endocrine glands might play a role in the production of the condition.

MATERIALS AND METHODS

Long-Evans adult, nulliparous female rats weighing 158–267 grams were used. Pregnancies were dated from the day spermatozoa were observed in the vaginal smear before 9 A.M. Body weights, red blood cell counts and hemoglobin determinations were recorded on the 1st, 13th, 19th and 22nd days of pregnancy in all experiments.

Blood samples were obtained from the tail of the rat. All red cell counts were made in duplicate and hemoglobin concentration was determined as acid hematin using a Fisher Electro-Hemometer. The color index was calculated according to the following formula:

$$\frac{\text{gm. Hb/100 cc. experimental rat blood}}{\text{gm. Hb/100 cc. normal rat blood}} \text{ divided by } \frac{\text{experimental red cell count}}{\text{normal red cell count}}$$

(Magner, 1938). The average of first day determinations for each experimental group was considered as normal in this work.

Five rats were used in each of 12 experimental groups. The hypophysis, ovaries, placentae and fetuses were removed separately and in all possible combinations. For a list of the various experiments, see Table 1. The average of the results for each group on the 13th, 19th and 22nd days was compared with the initial average, and the various group averages were compared at the end of the experiments. Statistical significances of the data were calculated according to the method of Snedecor (1946; Chap. 4) only on the results obtained on the 22nd day of pregnancy.

All operations were performed on the 13th day of pregnancy except when hypophysectomy and operations on the genital tract were combined; in these cases, animals were hypophysectomized on the 14th day. When both fetuses and placentae were to be removed, the animals were hysterectomized. Removal of the fetuses alone was accomplished through small incisions in the antimesometrial wall of the uterus. The cut edges of uterine muscle were approximated by single sutures. Ovariectomy was performed by the lumbar approach except in groups which also required removal of fetuses or hysterectomy.

Animals (except those hypophysectomized) were autopsied on the 22nd day of pregnancy. When placental tissue was present, the number and total

weight of the placentae were recorded. Incompletely hypophysectomized animals were not included.

OBSERVATIONS AND RESULTS

During normal pregnancy there was a decided diminution of red cell number and hemoglobin concentration per unit volume of blood which was noticeable on the 13th day and decreased progressively until the last day of pregnancy (Table 1, Group I). The decline of these two blood constituents remained parallel throughout gestation

TABLE 1. AVERAGE RED BLOOD CELL NUMBER (MILLIONS PER CUBIC MILLIMETER) AND HEMOGLOBIN (GRAMS PER 100 CUBIC CENTIMETERS) ON THE 1ST, 13TH, 19TH AND 22ND DAYS OF PREGNANCY, COLOR INDEX (C. I.) FOR EACH GROUP OF RATS AND THE AVERAGE WEIGHT OF ONE PLACENTA IN MILLIGRAMS. BLANK SPACES INDICATE ORGANS REMOVED.

Group	1		13		19		22		C.I.	Plac. wt.
	rbc	Hb	rbc	Hb	rbc	Hb	rbc	Hb		
I. AP+ovary+plac+fetus	8.88	15.1	7.88	13.7	7.16	11.7	6.18	10.4	0.99	558[1]
II. ——+ovary+plac+fetus	9.36	14.5	8.01	13.2	7.17	12.0	6.09	10.1	1.07	433[1]
III. AP+——+plac+fetus	9.69	14.8	8.56	13.9	8.14	13.3	7.57	12.4	1.07	253
IV. AP+ovary+plac+——	9.49	15.3	8.40	14.0	7.86	12.8	7.79	13.3	1.06	134
V. AP+——+plac+——	9.32	14.9	8.62	13.9	7.19	12.3	7.61	12.4	1.02	247
VI. AP+ovary+——+——	9.46	14.8	8.55	13.7	9.74	16.2	9.20	15.4	1.09	—
VII. ——+ovary+plac+——	9.18	14.9	8.24	13.9	7.35	12.1	6.61	10.9	1.02	105
VIII. ——+——+plac+fetus	9.18	14.6	8.76	14.0	6.20	9.6	4.36	7.0	1.00	377
IX. ——+——+plac+——	9.68	15.9	8.04	13.1	7.55	11.5	6.80	10.6	0.95	289
X. AP+——+——+——	9.47	15.0	8.38	14.2	8.88	15.2	9.30	15.5	1.05	—
XI. ——+ovary+——+——	9.54	14.4	8.64	14.2	9.27	16.0	9.38	15.0	1.05	—
XII. Non-pregnant, hypophysectomized			9.40	16.4	8.40	14.3[2]	8.02	12.9[2]	0.93	—

[1] From Leonard, S. L.: *Anat. Rec.* 91: 65. 1945.
[2] In this case, figures are for 6 and 9 days after hypophysectomy.

with the most rapid decrement occurring between the 13th day and the termination of pregnancy. The color index also indicated the parallel change in red blood cells and hemoglobin.

To investigate the effect of the pituitary gland on the blood picture during pregnancy, similar blood studies were conducted on hypophysectomized non-pregnant and pregnant female rats. In pregnant rats hypophysectomized for 9 days, the hemoglobin and red cell levels were diminished to the same extent as in normal pregnancy (Table 1). On the other hand, the red cell and hemoglobin levels of non-pregnant rats hypophysectomized for the same number of days decreased to only 8.02 millions and 12.9 grams respectively.

Blood studies were made on hypophysectomized pregnant rats after the normal time of parturition. It was found that within one week the red cell and hemoglobin levels rose consistently (6.09 to 7.47 millions and 10.1 to 11.6 gm.). This seemed to indicate that the anemia of pregnancy was more severe than the anemia resulting from hypophysectomy within comparable periods. Normal rats recover from the anemia of pregnancy shortly after parturition, often within one week (Beard and Myers, 1933), whereas the hypophysectomized rats were still anemic four weeks after delivery (7.63 millions and

11.3 gm.). The mean values for this group four weeks after the end of gestation corresponded to those of non-pregnant animals which had been hypophysectomized for the same length of time (6.92 millions and 11.3 gm.).

The removal of the ovaries in the rat usually interrupts pregnancy; the fetuses die but the placentae remain "alive" (Selye, Collip and Thomson, 1935; Huggett and Pritchard, 1945). The blood studies of ovariectomized rats showed that even in the absence of the usual complement of living fetuses there was a 21.9% drop in the red cell number and a 16.2% decline in hemoglobin. This reduction, however, was not as great as in normal pregnancy (30.4% and 31.1%). In two cases of ovariectomized animals, one malformed fetus was carried to term supporting the results of the experiment of Haterius (1936) who found that if all fetuses but one were eliminated and all placentae were left intact, the remaining fetus would be carried to the end of gestation. The blood picture in these two cases did not differ from that of the other ovariectomized animals.

When only the fetuses or when both fetuses and ovaries were removed on the 13th day of pregnancy, the results were not significantly different from those obtained in ovariectomized pregnant rats (Table 1; Groups III, IV, V). In all three of these groups an influence of the fetuses was lacking, and it appeared that the presence or absence of the ovaries was unimportant in relation to these blood constituents. The lack of influence of the ovaries was again demonstrated in Groups VII (ovaries and placentae remaining) and IX (placentae only remaining) (Table 1).

If only the placentae were retained (Group IX), the blood constituents were not significantly different from those of unoperated pregnant animals. *Placental tissue in the absence of the pituitary, fetuses and ovaries produced an anemia almost as severe as that of normal pregnancy.* A similar blood picture was observed in the group with retained placentae and ovaries (Group VII). The placenta, apparently, is the important organ in producing the anemia in these animals. Again, animals with placentae or placentae and ovaries remaining (Groups IX and VII) became anemic to a greater degree than those in which the pituitary was present in addition to the above organs (Groups V and IV). Thus, it seemed that the placenta adversely affected the composition of the blood and that the pituitary compensated somewhat for the action of placental tissue.

In the group of animals with placentae and fetuses intact and with hypophyses and ovaries extirpated (Group VIII), the mean red cell number and hemoglobin value were reduced considerably more than under any other experimental conditions. Frequently, animals of this group seemed in poor health. The absorption of toxic products from the necrotic fetal tissue may have caused this unhealthy condition especially pronounced in the hypophysectomized rats. Partial abor-

tion and the resulting loss of blood which occurred in this group may also have contributed to the severity of the anemia.

The results obtained from the study of hysterectomized animals proved that if the fetuses and placentae were removed (Group VI), the red cell number and hemoglobin concentration returned to their original levels within 9 days. This return to normal also occurred even if the ovary or pituitary had been removed in addition to the placentae and fetuses (Groups X and XI).

All placentae which were retained until the day of autopsy were weighed (Table 1). A wide individual variation in size and vascularity

TABLE 2. A SUMMARY OF THE AVERAGE BODY WEIGHTS ON THE 1ST, 13TH, 19TH AND 22ND DAYS OF PREGNANCY AND THE AVERAGE WEIGHT INCREMENT DURING GESTATION FOR EACH GROUP OF ANIMALS. THE AVERAGE WEIGHT OF ONE PLACENTA IS GIVEN FOR GROUPS IN WHICH SUCH DATA WERE OBTAINED. BLANK SPACES INDICATE ORGANS REMOVED.

Group	Body weight gm.				Increment		Plac. mg.
	1	13	19	22	gm.	%	
I. AP+ovary+plac+fetus	242	261	277	314	72	29.8	558[1]
II. ——+ovary+plac+fetus	233	263	252	277	44	18.9	433[1]
III. AP+——+plac+fetus	213	242	238	235	22	10.3	253
IV. AP+ovary+plac+——	214	242	234	239	25	11.7	134
V. AP+——+plac+——	211	249	235	237	26	12.3	247
VI. AP+ovary+——+——	214	236	218	215	1	0.47	—
VII. ——+ovary+plac+——	203	234	211	208	5	2.5	105
VIII. ——+——+plac+fetus	218	242	226	219	1	0.45	377
IX. ——+——+plac+——	203	231	202	202	−1	−0.49	289
X. AP+——+——+——	220	247	235	237	17	7.7	—
XI. ——+ovary+——+——	221	262	223	218	−3	−1.35	—
XII. Non-pregnant, hypophysectomized	—	223	202	197	−26	−11.7	—

[1] Data from Leonard, S. L.: *Anat. Rec.* 91: 65. 1945.

was observed. It was impossible to judge the physiological activity of these organs from the gross appearance. The average weight of one placenta was somewhat less in hypophysectomized rats than in normal pregnant animals. In ovariectomized rats the average weight of one placenta was reduced to less than 50% of the normal weight. A similar reduction occurred when both fetuses and ovaries were removed. If only fetuses were eliminated, the decrease in mean placental weight was even greater. Likewise, wide variations were encountered in the groups of hypophysectomized rats with fetuses or ovaries or both removed. The smallest placentae were found in the group with pituitary and fetuses removed. With the present available information, it is difficult to correlate placental weight with function as reflected in the blood picture.

In no group of operated pregnant animals did the body weights fall significantly below the original level (Table 2). All animals gained more weight between the 1st and 13th days of pregnancy than could

be accounted for by the estimated weight of the fetuses and placentae. Between the 13th day and the end of gestation, the group of normal pregnant animals also gained more weight than could be explained by the weight of the fetuses and placentae. In hypophysectomized pregnant rats, however, the weight increment during this period was not greater than the estimated weight of fetuses and placentae.

In the other experiments in which the pituitary gland remained (Groups III, IV, V, X), the body weight did not return to the original level after the 13th day. The final body weight in Group VI (hypophysis and ovaries remaining), however, did return to the original weight.

The pregnant animals of the groups mentioned above (III, IV, V) decreased in weight after the removal of the ovaries and fetuses but not to the level of the first day of gestation. At first sight, this might be attributed to the presence of the pituitary and placenta. In Group X (pituitary only remaining), however, the body weight failed to return to the original level whereas in Group VI (pituitary and ovaries remaining) the body weight did return. Thus, no completely satisfactory explanations can be made on body weight changes in some of the experiments without further investigation.

DISCUSSION

From the results of this series of experiments it is possible to show a relationship of the pituitary, ovaries, placenta and fetus in the production of anemia in the pregnant rat. Analysis of the data indicates that the placenta is probably the most important single factor in the development of this condition. Whenever the placenta remained, a significant decrease in erythrocyte count and hemoglobin value occurred. The degree of the anemia was not always the same in the presence of one or more of the other three factors considered in this study, and an attempt will be made to account for these differences.

A living fetus may be responsible, to some extent, for the anemia of pregnancy in the rat (Groups I, II). The placentae reached their maximum weight and presumably their maximum vascularity and activity only when a living fetus was present (Table 1). Under such conditions its anemia-inducing capacity should also be at its height. Even though there did not appear to be a correlation between the size of the placentae and the degree of activity in all experiments, the exceedingly large development of the placentae in the presence of a living fetus might explain, in part, the severity of the anemia in these groups. The placentae continued to grow in the absence of the fetus and ovary, but dead fetuses did not appear to be conducive to the extensive development of placental tissue as living fetuses were.

The pituitary gland has been shown to be unessential for the continuation of normal pregnancy after the 11th day of gestation

(Pencharz and Long, 1931; 1933). Within 9 days, hypophysectomy of non-pregnant animals affected the blood picture (Group XII), but in pregnant rats hypophysectomized for the same number of days there was an anemia no more severe than in unoperated pregnant animals. In Group IX (only placentae remaining), however, the anemia which followed may actually be a summation of that induced by the placenta and the absence of the pituitary. This group showed a more severe anemia than when both the pituitary and placenta remained (Group V).

From the experiments of other workers, it has been noted that the hypophysis, either directly or through the other endocrine glands, acts to increase the red blood cell number and hemoglobin concentration (Flaks, Himmel and Zlotnik, 1937; 1938; Querido and Overbeek, 1938; 1939; Meyer, Thewlis and Rusch, 1940). The pituitary, therefore, would tend to counteract the influence of the placenta. It appears from the data presented in this paper that the hematopoietic-inducing activities of the hypophysis were not great enough to compensate for the action of a fully functional placenta as found in normal pregnancy. When only placentae with a reduced activity remained, however, i.e., in the absence of a living fetus, the anemia may be partially counteracted by the pituitary.

In all cases when the placentae were eliminated and the pituitary left intact, the blood picture returned to normal at least within 9 days. The blood composition also became normal in the same number of days in that group from which both placentae and hypophysis were removed (Group XII). These facts are an indication that the anemia of pregnancy in the rat may be the result of an hydremic effect. This point is discussed later.

The ovary apparently played no direct role in the development of anemia during the last half of pregnancy. It is important in relation to the anemia, however, in that it sustained living fetuses, and, hence, induced normal growth of the placenta. A comparison of Groups VII (placentae and ovaries remaining) and IX (only placentae remaining) demonstrated the lack of importance of the ovary itself. The severity of anemia was the same in both cases. Similar evidence is noted when a comparison is made between Group IV (hypophysis, ovaries and placentae remaining) and V (hypophysis and placentae remaining).

The progressive nature of the anemia of pregnancy in the rat was noted in the experiments in all groups that developed the greatest anemia. It is remarkable that the limits to which it developed should be so similar except in Group VIII when other factors (excessive bleeding) were encountered. By the 13th day of pregnancy consistently lowered red cell count and hemoglobin were noted.

The nature of the anemia of pregnancy in the rat has not been established by these experiments since no studies were made on the

hematopoietic organs or on the blood plasma concentration. Some evidence was obtained indicating that hydremia may be the important factor. In all experimental groups the color index varied only slightly from unity. Simple blood dilution can most easily explain the constancy of this factor. Also, after removal of the placentae (Groups VI, X, XI), the blood picture very rapidly returned to normal. Water exchanges in the body can take place with amazing rapidity whereas it might take longer to affect the hematopoietic organs. This became more obvious when it was observed that in spite of hypophysectomy and placental removal the blood composition returned to normal before the effects of pituitary removal were exerted fully.

Other investigations on the anemia of pregnancy in the rat have shown that hydremia must be considered as a possible explanation. A decided drop in the concentration of serum proteins occurred during pregnancy (Sure, Kik and Walker, 1929). The water content of whole blood increased 3.5% during gestation, and the refractive index declined after the 16th day of gestation (Van Donk, Feldman and Steenbock, 1934). The fact that the refractive index did not begin to decrease until after a diminution of hemoglobin had been observed does not obviate the possibility that other factors may also be involved.

With regard to the mechanism of the action of the placentae, one may only conjecture. The anemia-inducing effect of the placenta may be the result of a competition between the growing placenta and body tissues for the basic materials necessary for red cells and hemoglobin. This presupposes that the hematopoietic organs would be affected by the placenta and hydremia would be of little consequence. Also, there is a possibility that the enlargement of the uterine blood vessels and those of related parts would require more fluid to maintain blood volume. An augmentation of blood volume could be accomplished most easily by the movement of water into the plasma. In all cases where the placentae were present, extreme vascularity of the uterus was observed. Finally, the placenta might produce a chemical substance of such a nature as to cause a shift of water into the blood. This last possibility offers a suggestion of a hormonal function of the placenta.

The fact that the placentae of rats and other mammals produce several hormones suggests that the anemia may be induced by hormonal action. It has been demonstrated that the placenta produces hormones of several kinds some of which, in pure form, induce anemia, i.e., estrogens. Studies on various mammals indicate that estrogen, progesterone and an anterior pituitary-like hormone are produced by the placenta. Specifically, it has been shown that the placenta of the rat produces a substance stimulating mammary gland development (Leonard, 1945) and another maintaining the corpora lutea (Astwood

and Greep, 1938). It is possible that these hormones, or others as yet undetected, may, in some manner, affect the red cell and hemoglobin levels during pregnancy.

In conclusion, it appears that the anemia of pregnancy in the rat develops primarily under the influence of the placenta and partially or indirectly under the influence of the fetus. The hypophysis acts, under certain conditions, to compensate for the anemia-inducing

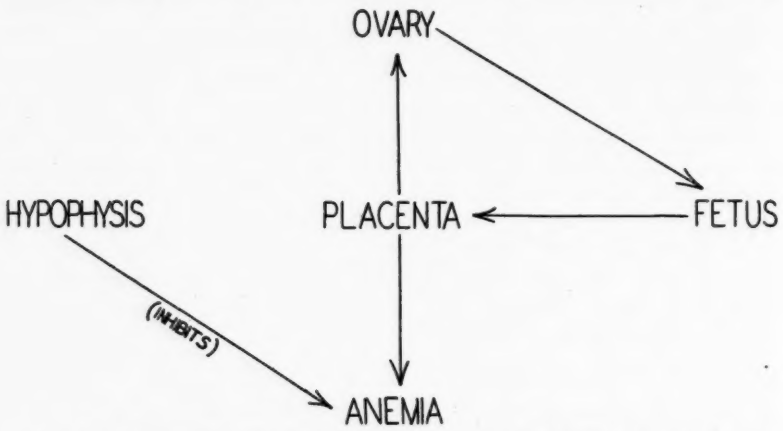


FIG. 1. A diagrammatic representation of the possible relationships of certain organs during the last half of pregnancy in the rat as they affect anemia.

Placenta: main factor in anemia; keeps ovary in luteal phase of activity
Fetus: essential for greatest growth and function of placenta
Ovary: maintains living fetuses
Hypophysis: counteracts anemia; action on placenta and ovary unknown

action of the placenta. The effect of the placenta may be produced merely by certain metabolic activities, or its influence may be exerted by the hormones it produces acting either directly on the erythropoietic organs or by the production of hydremia. Before the method of action can be ascertained, studies must be made on the specific gravity of the blood, on the refractive index of the plasma and on the effect of extracts of the placenta of pregnant rats in a series of experiments similar to those presented in this paper.

A diagram of the possible relationships of the pituitary, ovary, placenta and fetus to anemia of pregnancy is presented in Figure 1.

SUMMARY

Studies were made on the effect of removal of the placenta, ovary, fetuses and hypophysis, individually and in all combinations, during the last half of pregnancy on the anemia of pregnancy in the rat. The placenta alone (in the absence of the pituitary, ovary and fetus) was capable of producing a definite and consistent anemia almost equal

to that found in normal pregnancy. Although the mechanism of the placental action is unknown, a possible hormonal mechanism is discussed. It was not possible to completely correlate placental weight and the degree of anemia in the different experimental groups. Neither the ovary nor the living fetus seemed to have any direct influence on the production of anemia. The effect that was indicated might be the result of a more active or functional placenta induced by the presence of the living fetus. The removal of the pituitary gland did not noticeably influence the anemia of pregnancy within the period of the experiment, but the post-hypophysectomy anemia became obvious after parturition. When the pituitary was removed in animals with only retained placentae, the reduction of red cells and hemoglobin was greater than in animals with the placentae and hypophysis intact. The blood picture of pregnant animals which were hysterectomized returned to normal within 9 days. Suggestive evidence indicated that hydremia may be the cause of the anemia of pregnancy.

REFERENCES

ASTWOOD, E. B., AND R. O. GREEP: *Proc. Soc. Exper. Biol. & Med.* 38: 713. 1938.
BEARD, H. H., AND V. C. MYERS: *Am. J. Physiol.* 106: 449. 1933.
CRAFTS, R. C.: *Endocrinology* 29: 596. 1941.
FLAKS, J., I. HIMMEL AND A. ZLOTNIK: *Presse méd.* 45: 1261. 1937.
FLAKS, J., I. HIMMEL AND A. ZLOTNIK: *Presse méd.* 46: 1506. 1938.
GORDON, A. S., AND H. A. CHARIPPER: *Anat. Rec.* 85: 312. 1943.
HATERIUS, H. O.: *Am. J. Physiol.* 114: 399. 1936.
HUGGETT, A. ST. G., AND J. J. PRITCHARD: *Proc. Roy. Soc. Med.* 38: 261. 1945.
LEONARD, S. L.: *Anat. Rec.* 91: 65. 1945.
MAGNER, W. *A Textbook of Hematology.* P. Blakiston's Son and Co., Inc. Philadelphia. 1938.
MEYER, O. O., E. W. THEWLIS AND H. P. RUSCH: *Endocrinology* 27: 932. 1940.
MITCHELL, H. S., AND L. MILLER: *Am. J. Physiol.* 98: 311. 1931.
PENCHARZ, R. I., AND J. A. LONG: *Science* 74: 206. 1931.
PENCHARZ, R. I., AND J. A. LONG: *Am. J. Anat.* 53: 117. 1933.
QUERIDO, A., AND G. A. OVERBEEK: *Arch. internat. de pharmacodyn. et de thérap.* 59: 370. 1938.
QUERIDO, A., AND G. A. OVERBEEK: *Arch. internat. de pharmacodyn. et de thérap.* 61: 475. 1939.
SELYE, H., J. B. COLLIP AND D. L. THOMSON: *Proc. Soc. Exper. Biol. & Med.* 30: 589. 1933.
SELYE, H., J. B. COLLIP AND D. L. THOMSON: *Endocrinology* 19: 151. 1935.
SNEDECOR, G. W.: *Statistical Methods*, 4th Edition. The Iowa State College Press. Ames. 1946.
STEINGLASS, P., A. S. GORDON AND H. A. CHARIPPER: *Proc. Soc. Exper. Biol. & Med.* 48: 169. 1941.
SURE, B., M. C. KIK AND D. J. WALKER: *J. Nutrition* 1: 299. 1929.
VAN DONK, E. C., H. FELDMAN AND H. STEENBOCK: *Am. J. Physiol.* 107: 616. 1934.
VOLLMER, E. P., AND A. S. GORDON: *Endocrinology* 29: 828. 1941.

ESTROGENIC ACTIVITY OF RABBIT WHOLE BLOOD DETERMINED BY THE INTRAVAGINAL PELLET METHOD

BORIS KRICHESKY, PH.D., AND S. J. GLASS, M.D.
Department of Zoology, University of California
LOS ANGELES, CALIFORNIA

A SIMPLIFIED method for the bioassay of estrogens in the blood is urgently needed. Several technics have been suggested thus far. That of Frank and Goldberger, (1935), required the use of 40 cc. of whole blood which after chemical extraction will yield in the normal human subject only 1 mouse unit by the standard Allen Doisy technic. Thus, the large volume of blood necessary for a positive response invalidates this method for wholesale testing.

The paravaginal estrogen assay method of Hartman and Littrell (1945) is reported by these workers to be extremely sensitive, yielding positive responses with as little as 0.02 cc. whole blood. However, Lloyd *et al.* (1946) in a similar study, found that "the inconstancy of results and lack of sensitivity of this method preclude its use as a quantitative assay method."

Before the paravaginal assay method was reported we became interested in the practical aspects of the bioassay of blood by means of intravaginal pellets of whole blood described by Albrieux (1941). This method is based on the observations of Lyons and Templeton (1936) who noted strikingly increased sensitivity of the spayed adult rodent to the intravaginal instillation of urinary estrogenic extracts. This increased sensitivity of the vaginal mucosa provides a ready means of assaying low concentrations of estrogens in blood and urine. Thus the method lends itself to the study of estrogen metabolism in small laboratory animals as well as in the human subject. To test the accuracy and usefulness of the intravaginal pellet technic of Albrieux, whole blood from rabbits and a few women were studied. Minor modifications of the method of Albrieux were applied in the present report.

MATERIALS AND METHODS

The rabbits used in this study were all young adults of the Dutch breed raised in our own laboratories. These animals were isolated from 4 to 6 weeks to eliminate pseudopregnancy. Ten cc. of whole blood was collected from the marginal ear veins, poured into a 15 cm. petri dish and dried for 48 hours in a vacuum desiccator over calcium chloride. The dried blood was

Received for publication January 20, 1947.
* Aided by grants from the Ayerst, McKenna and Harrison, Ltd., Rouses Point, New York and the Board of Research, University of California.

then scraped from the dish, ground to a fine powder, placed in a weighed open vial and stored in a vacuum dessicator for 24 hours. The vial containing the dried powdered blood was then weighed. Pellets of the whole blood were made by adding 10% by weight of sesame oil to act as a binder. The mixture was then hammered into pellets of varying length. The pellets were weighed to the nearest 0.5 mgm. and selected for use. Each pellet used was cut into two approximately equal parts and introduced into the vagina of carefully selected ovariectomized young adult rats by a glass trochar. One part of each weighed pellet was placed intravaginally into the same rat on each of two successive days in order to simulate divided dosage for increased sensitivity.

Human blood used in these studies was measured and treated in the manner described for rabbit blood. It was found that 10 cc. of rabbit or human blood, when dried in the manner described, weighed between 2.012 and 2.203 gms. with an average of 2.100 gms. The minor variations in the weight of the different blood samples may be accounted for by the difficulty in measuring accurately the 10 cc. of blood before and after clotting takes place.

All rats used were of the Wistar strain from 3 to 10 months of age. Approximately 3 weeks after ovariectomy, all animals were given a single subcutaneous injection of 50 I.U. estrone in oil.[1] Vaginal smears were taken 48 and 72 hours after injection and rats failing to show strong positive estrus were discarded. Ten days later the remaining animals were given a total of 0.5 I.U. estrone in oil intravaginally divided into two equal doses and administered 24 hours apart. Those animals failing to respond with a strong positive estrus smear were discarded. Only those which responded to this dose were used in subsequent studies. These assay animals were used not oftener than once in 2 weeks and 7 days before each assay, they were primed with 1.5 I.U. estrone given subcutaneously. The sensitivity of our colony was then determined by the installation of a solution of estrone in sesame oil intravaginally, giving a total of 0.05, 0.10, 0.15, 0.20 and 0.30 I.U. in two divided doses.

Ten rabbits, 5 intact and 5 castrate, provided the blood for these experiments. In addition one normal adult rabbit was given a single subcutaneous injection of 1500 I.U. of crystalline estrone and 10 cc. of blood was drawn from it 24 and 48 hours later for estrogenic assay. Four samples of human blood were also used for determination of estrogenic activity.

RESULTS

The pertinent data on the sensitivity of our animals are given in Table 1. These data indicate that a 50% response is obtained with a dose of estrone slightly less than 0.1 I.U. If these data are plotted it is found that the points at the 3 doses of 0.10, 0.15, and 0.20 I.U. fall on a straight line as shown in Figure 1, the doses at the two extremes failing to show a linear relationship. Calculations of the estrogenic activity of whole blood were made, whenever possible, only from data falling within the range of the straight line portion of the curve.

[1] We wish to thank the Eli Lilly Co., and the Schering Corporation for the estrone supplied to us.

The data obtained on blood studies are given in Table 2. Only those animals with vaginal smears showing numerous cornified epithelial cells were considered positive reactions.

The data show that the calculated estrogenic activity (estrone

TABLE 1. INTRAVAGINAL ASSAY OF CRYSTALLINE ESTRONE IN OIL SOLUTION

No. of animals	I.U. Applied	% positive
20	0.05	30
20	0.10	55
20	0.15	70
20	0.20	85
12	0.30	91
22	Sesame Oil	0

equivalent) of pellets of whole dried blood varies from 210 I.U. to 530 I.U. (in 1 case) per 100 cc. of whole blood in normal, nonpregnant, non-pseudopregnant rabbits. The average for the group is 308 I.U. estrone equivalents per 100 cc. Among the 5 ovariectomized animals, the level of blood estrogen falls, averaging only 150 I.U. The varia-

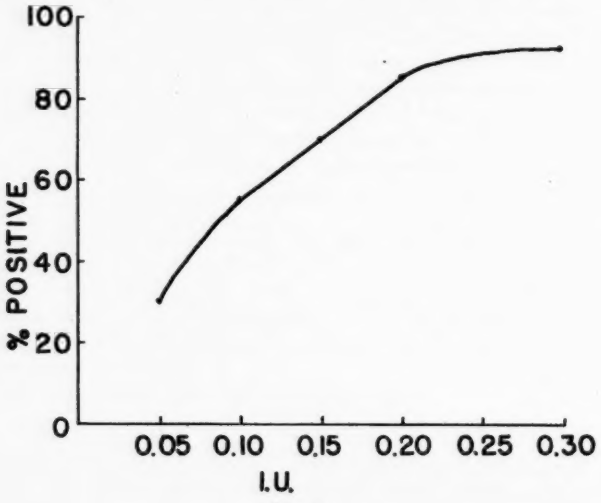


FIG. 1

tion is from 120–210 I.U. per 100 cc. whole blood. The value obtained from one castrate animal fell within the range of the normal intact animals. Similarly the value for one of the normal intact animals fell within the range of the castrate group.

Assay of the rabbit blood samples after subcutaneous injection of 1500 I.U. estrone in oil give values of 283 and 357 I.U. 100 cc. of whole

TABLE 2. ESTROGENIC ACTIVITY OF WHOLE BLOOD DETERMINED BY INTRAVAGINAL PELLETS

Specimen no.	Source of blood	Condition of donor	Wt. of pellets in mgs.	No. of test animals	% Positive	Approx. I.U. per 100 cc. (calc.)
1	Rabbit	Normal	5	7	42	
			10	6	66	280
			30	6	83	
2	Rabbit	Normal	10	10	60	
			20	10	80	240
			40	10	90	
3	Rabbit	Normal	3	6	33	
			10	6	66	280
			20	7	71	
4	Rabbit	Normal	5	8	25	
			15	8	50	210
			30	8	86	
5	Rabbit	Normal	3	11	45	
			13	10	90	530
					Average	308
6	Rabbit	Castrate	5	4	25	
			10	5	40	150
			25	5	80	
7	Rabbit	Castrate	6	14	36	
			20	9	90	210
8	Rabbit	Castrate	15	6	50	
			40	6	83	120
			70	8	90	
9	Rabbit	Castrate	10	7	42	
			20	6	83	150
			60	7	100	
10	Rabbit	Castrate	5	10	30	
			15	10	50	120
					Average	150
A	Human	Amenorrhea	5	10	60	
			20	12	83	480
			40	10	90	
B	Human	Amenorrhea	6	10	60	
			20	10	90	400
C	Human	Climacteric	5	10	30	
			20	10	70	200
			40	10	90	
D	Human	Surgical Castrate	5	9	23	
			15	10	60	160
			30	10	90	
I[2]	Rabbit	24 hrs. post injection	10	6	66	
			20	7	100	283
			40	7	100	
II[2]	Human	48 hrs. post injection	10	8	75	
			20	8	88	357
			40	8	88	

[2] This animal was given a single injection of 1500 I.U. estrone in oil subcutaneously.

blood. These values fall within the normal range and probably are not significant.

The four samples of human blood gave the following results: two from women with amenorrhea showed values which may be significantly higher than those obtained from a climacteric or from a surgical castrate (see Table 2). That the method is sufficiently accurate to give reasonably good reproducible results is indicated by the data in Table 3. Two normal and 2 castrate blood samples were

TABLE 3. REPEATED ASSAY OF WHOLE BLOOD PELLETS BY THE INTRAVAGINAL METHOD

Specimen no.	Source of blood	Donor	Assay value 1st run	Assay value 2nd run
2	Rabbit	Normal	240	280
3	Rabbit	Normal	280	260
8	Rabbit	Castrate	110	150
9	Rabbit	Castrate	150	150

assayed a second time 3 months after their first, in a different group of rats. The calculated estrogenic activity in these was nearly the same as in the original assay.

DISCUSSION

Comparison of our data with that of Albrieux reveals interesting similarities and differences which merit discussion.

Our rat colony showed essentially the same sensitivity to crystalline estrone in oil intravaginally as that observed by Albrieux, in that 0.10 I.U. of estrone induced cornification in 55 per cent of our animals and in 58 per cent in those of Albrieux. In our hands the intravaginal assay of crystalline estrone in oil yielded results with only an 8 per cent error. It is interesting to note in this connection that Lyons & Templeton (1936) reported a greater sensitivity to crystalline estrone but their preparations were in aqueous solution in contrast to our oily solution.

Albrieux reported values of 300–500 R.U./100 cc. blood in cases of amenorrhea whereas we obtained 400 and 480 I.U. in two similar cases studied. But it must be noted that Albrieux stated that "1 biologic rat unit . . . is equivalent to 0.10 unit of the international standard of estrone."

If we interpret Abrieux's data correctly our values in I.U. are approximately equivalent to the R.U. described by him. If this be true, then the data on sensitivity as well as those of whole blood assays in cases of amenorrhea agree fairly well.

Although the number of normal and castrate rabbits reported here is small, the average estrogenic values obtained in the two groups is significantly different, viz: an average value of 308 I.U. as compared with 150 I.U. respectively. It is difficult to reconcile the presence of definite estrogenic activity in castrate rabbits (120–200 I.U.) and in castrate human blood (160 I.U.) in our experiment with the absence of such activity in human castrates as reported by Albrieux. The data in Table 2 shows that 90 per cent of the test animals exhibited cornification with 30 mg. pellets of either human or rabbit castrate blood whereas Albrieux reports no such activity with pellets of 40–60 mgm. of human castrate blood. The fact that both rabbit and human castrate blood shows a similar range of estrogenic activity in our test animals suggests to us that such activity may be explained by (1) extragonadal endogenous estrogen secretion or (2) other hitherto unidentifiable substances in blood which are potentially capable of inducing vaginal cornification. Since estrogenic activity has frequently been reported to be present in the urine of castrate women, it is not surprising to find such activity in the blood. These estrogens may well be of adreno-cortical origin. Since we obtained vaginal cornification with castrate blood pellets the absence of a foreign body reaction reported by Albrieux must be reconsidered. Our data indicate that the circulating blood in an intact female rabbit possesses approximately twice the estrogenic activity of blood from an ovariectomized animal. If it is assumed that the activity obtained with castrate blood may be due to a foreign body reaction, then the difference between this and the activity obtained in normal rabbit blood may represent the true level of circulating estrogens.

The absence of any significant rise in the blood estrogen level after the subcutaneous injection of 1500 I.U. into one normal rabbit is not surprising. Because of prompt hepatic inactivation or of other threshold mechanisms, the normal animal will excrete at best only 10–20 per cent of the injected dose. It is suggested, therefore, that the injected hormone may be inactivated rapidly either by conjugation or by degradation.

SUMMARY AND CONCLUSIONS

A modified Albrieux method of assaying blood estrogens is presented. The results obtained indicate that there is approximately twice the estrogenic activity in whole blood of normal rabbits as in blood from castrate rabbits. Similar activity was found in the blood of two amenorrhoeic, one castrate and one climacteric woman. Bearing in mind the fact that there may be present in castrate blood substances capable of inducing vaginal cornification, the method here described may be applied in further studies of estrogenic activity in blood.

Inasmuch as non-specific or otherwise unidentifiable compounds in the blood pellets seem to induce vaginal cornification, a method is suggested whereby the true estrogenic activity may be estimated.

REFERENCES

ALBRIEUX, A. S.: *J. Clin. Endocrinol.* 1: 889–893. 1941.
FRANK, R. T., AND M. A. GOLDBERGER: *Proc. Soc. Exper. Biol. & Med.*, 32: 1663. 1935.
HARTMAN, C. G., AND J. LITTRELL: *Science* 102: 178. 1945.
LOYD, C. W., W. F. ROGERS AND R. H. WILLIAMS: *Endocrinology* 39: 256. 1946.
LYONS, W. R., AND H. TEMPLETON: *Proc. Soc. Exper. Biol. & Med.* 33: 587. 1936.

THE EFFECT OF THE PROSTATE AND SEMINAL VESICLE ON THE PRODUCTION OF HYALURONIDASE BY RAT TESTES HOMOGENATE[1]

S. L. LEONARD, P. L. PERLMAN[2] AND R. KURZROK
From the Department of Zoology, Cornell University
ITHACA, N. Y. AND NEW YORK, N. Y.

HYALURONIDASE, an enzyme formed by the mammalian sperm and also the testes, is thought to play a role in the fertilization process by its action in dispersing the follicle cells from recently ovulated mammalian ova preceding sperm entry (McClean and Rowlands, 1942; Fekete and Duran-Reynals, 1943; Leonard and Kurzrok, 1945).

In the course of some experiments upon the determination of this enzyme, it was observed that the concentration in the ejaculate of the rat, obtained from the uterus of the female shortly after breeding, was inordinately high as compared with the enzyme concentration from extracts of testes. Allowing for the fact that non-enzyme producing tissue in the testes is present, two possible explanations were considered: (a) since the extracts of the testes containing the enzyme were made at 10° C., this low temperature might account for the low enzyme concentration, (b) the secretions of the prostate and seminal vesicles might favor the production of the enzyme by furnishing a better environment for the continued production of hyaluronidase. The assumptions were tested and preliminary results are now reported.

METHODS AND RESULTS

Methods of assaying hyaluronidase and the preparation of tissue extracts used in these studies have recently been published (Leonard, Perlman, and Kurzrok, 1946).[3] The enzyme unit is expressed as the *turbidity reducing unit* (TRU). In each of the experiments below, the pooled tissues of 3–4 rats were required to obtain sufficient material for assay.

Testes of adult rats were homogenized and incubated at 37° C. for 8 hours before continuing the preparation of the extract. Assays for

Received for publication January 28, 1947.
[1] Acknowledgment is made to the Trustee-Faculty Committee on Research for support of this work.
[2] Fellow of the Schering Corporation.
[3] Two errors in methods appeared in this reference: page 263, paragraph 2 should read " . . . 3.0 ml. pH 4.2 acetate buffer . . . "; table I should read "incubate 30 minutes, chill for 5 minutes; add following volume (ml.) to each tube."

hyaluronidase of these testes gave enzyme concentrations of 30–40 TRU per gram of testes whereas those kept in the ice box at 10° C. yielded 10–20 TRU per gram of tissue. It was therefore apparent that at the higher temperatures, increased amounts of the enzyme were produced by the testicular tissue.

To determine the effects of the accessory glands on hyaluronidase production, three simultaneous assays were made on (1) homogenized testes, (2) homogenized seminal vesicles and prostates, (3) combination of (1) and (2) in the proper ratio, i.e., two seminal vesicles and one prostate for each pair of testes. The extracts were prepared so that

TABLE 1. HYALURONIDASE ASSAYS OF EXTRACTS OF TISSUE HOMOGENATES

Tissue	TRU (per gram of tissue)	
	Exper. 1	Exper. 2
Testes+Prost.+Sem. Ves.	70	24*
Testes	40	11
Prost.+Sem. Ves.	10	10
Testes+Prost.	35	66
Testes	30	60
Prost.	15	20
Testes+Sem. Ves.	88	80
Testes	45	35
Sem. Ves.	10	5

* Incubation of tissue homogenate before extraction at 10° C., the others at 37° C.

one gram of tissue was equivalent to one cubic centimeter of extract. The tubes were left at 10° C. for eight hours. Assays of the resulting extracts gave the following concentrations per gram of tissue: testes = 11 TRU; prostate+seminal vesicle = 10 TRU; combination = 24 TRU. Repeating this experiment, but incubating at 37° C. for eight hours gave the following results per gram of tissue: testes = 40 TRU; prostate+seminal vesicle = 10 TRU; combination = 70 TRU. Thus in both instances the testes were able to produce more enzyme because of something added from the accessory glands.

To determine which accessory gland played a part in this augmentation of enzyme production similar experiments were set up using the seminal vesicles and prostates alone. The results are given in Table 1. It is apparent that the seminal vesicles contribute a factor to the testes cells which enable them to produce more enzyme in a given period than if prostatic tissue was added. No augmentation of enzyme production occurred with prostatic tissues and it seemed that the augmentation obtained with both accessories plus testes was accountable on the basis of the contribution from the seminal vesicles alone.

The presence of some hyaluronidase in the seminal vesicles and prostate was disconcerting at first when abundant evidence indicated

that it was the sperm or germinal epithelium of the testes which produced the enzyme (McClean and Rowlands, 1942; Leonard, Perlman, and Kurzrok, 1946; Duran-Reynals, 1942). From certain observations it appeared to us that the enzyme diffused from the sperm which were present in the distal portion of the ductus deferens. Since the prostate is in more intimate contact with the ductus deferens throughout a greater length than are the seminal vesicles, this offered a possible explanation for the higher concentration of the enzyme which was found in the prostate. Using the rat-ova test (Leonard and Kurzrok, 1945), it was not possible to detect any enzyme in prostatic tissue obtained from castrate immature rats treated with testosterone and in which, of course, no spermatozoa were ever present.

DISCUSSION

These results may explain in part the reason for higher enzyme concentration in the ejaculate as compared with the testes. Part of the explanation also may lie in the fact that extraneous non-enzyme producing tissue of the testes would make the enzyme concentration lower per gram of tissue and that fully formed sperm may be more efficient producers of the enzyme.

The finding that the addition of seminal vesicle tissue to the testes homogenate greatly increased the production of the enzyme complements the work of Mann (1946) who has recently shown that the primary energy source for sperm is fructose. This sugar was found to occur in its highest concentration in the seminal vesicles or prostate, according to the mammalian species. Therefore it would seem that one of the functions of the seminal vesicle secretion is to increase the hyaluronidase output of spermatozoa in the rat.

SUMMARY

Addition of rat seminal vesicle tissue to rat testes homogenate resulted in an increased production of hyaluronidase as compared with testes homogenate alone.

Prostatic tissue did not exhibit this effect although both the prostate and seminal vesicle normally contain hyaluronidase. It is thought that this hyaluronidase diffuses into these organs.

It is concluded that one of the functions of the seminal vesicle secretion may be to increase the hyaluronidase output of spermatozoa in the rat.

REFERENCES

Duran-Reynals, F.: *Bact. Rev.* 6: 197. 1942.
Fekete, E., and F. Duran-Reynals: *Proc. Soc. Exper. Biol. & Med.* 52: 119. 1943.
Leonard, S. L., and R. Kurzrok: *Endocrinology.* 37: 171. 1945.
Leonard, S L., P. L. Perlman and R. Kurzrok: *Endocrinology.* 39: 261. 1946.
Mann, T.: *Biochem. J.* 40: 481. 1946.
McClean, D., and I. W. Rowlands: *Nature, London* 150: 627. 1942.

THE EFFECTS OF GONADECTOMY AND THE ADMINISTRATION OF TESTOSTERONE PROPIONATE ON THE GROWTH OF ANTLERS IN MALE AND FEMALE DEER[1]

GEORGE B. WISLOCKI, JOSEPH C. AUB, AND CHARLES M. WALDO
The Harvard Medical School
BOSTON, MASSACHUSETTS

SEVERAL of us at the Harvard Medical School have been interested for some years in the study of the antlers of deer. We have attempted more especially to analyze by experimental means the factors controlling the annual renewal, growth, and shedding of the antlers. For this purpose, we have availed ourselves of a group of Virginia or white-tailed deer kept in captivity at the Wildlife Research Center, Delmar, N. Y.[2]

The present investigations have been concerned with the effects of castration upon the growth of the antlers of deer at different ages and times of the year, as well as with the effects of testosterone propionate administered to both normal and castrated male and female deer.

MATERIAL AND METHODS

The experimental procedures were carried out on twenty-one Virginia deer (*Odocoileus virginianus borealis*) ranging in age from two months up to nine years. The animals had been captured as fawns in the first months of life and were brought up in captivity. They were kept in a series of commodious paddocks especially designed for the maintenance of deer for experimental purposes. The paddocks were provided with unheated shelters and appropriate food and drinking troughs.

In the laboratory of the Research Center, adequate facilities existed for

Received for publication February 4, 1947.
[1] These investigations have been supported by grants from the Milton Fund of Harvard University and from the Elizabeth Thompson Science Fund. This is publication 629 of the Cancer Commission of Harvard University.
[2] The investigations reported here have been carried out at the Wildlife Research Center, Delmar, N. Y., under an agreement with the Conservation Department of the State of New York. A number of the executives and younger scientists of that organization have been particularly helpful in giving advice and rendering assistance in the course of the work. We feel greatly indebted to Messrs. Gardiner Bump, Glenn Morton and E. L. Cheatum who were at the Research Center when the investigations were started. The work was continued with the cooperation of Messrs. David B. Cook, William Severinghaus and Robert W. Darrow to whom we owe our gratitude. We are indebted also to Mr. Cary Stott for the excellent care which he has given the animals and for the frequent observations which he has made on them.

operating on deer under aseptic conditions. Transfer of the deer from the paddocks to the operating room was facilitated by the construction of especially designed narrow box traps into which a deer could be driven and a trapdoor closed behind it.

The animals were released from the box trap at the laboratory and were restrained routinely by three or four men until nembutal had been given intravenously by the veins of the ears. The ear was shaved with an electric razor and nembutal was introduced into a vein by means of a thirty cubic centimeter syringe and twenty gauge needle until anesthesia was achieved. This occurred within a few minutes with doses ranging from 650 to 780 mg. for 4-months' old fawns up to 1600 to 1900 mg. for adult animals. If, in the course of the subsequent operation, the anesthesia became light and the animals began to move, further doess of nembutal were given, several adult deer eventually receiving as much as 2700 mg. before the operation was completed.

The postoperative recovery was almost invariably uneventful. None of the animals showed any infection at the sites of operation, but some of them eventually lost portions of an ear due to local vascular occlusion at the sites of injection of nembutal.

The first of these operations, which consisted in the removal of the testes or the ovaries, was undertaken in the fall of 1942 and the remainder in the following years.[3] A number of the experimental animals have now been alive for nearly four years.

The giving of testosterone to our experimental deer led to the devising of a special technique for administering it. In view of the frequency with which it would be necessary to give hypodermic injections and the trouble involved in first catching and throwing the deer, administration of testosterone by injection was ruled out. The introduction of pellets by surgical implantation after anesthetizing an animal, a procedure which we used at first, did not offer a less complicated alternative. Instead, it was found possible to shoot pointed slugs of testosterone into the thick muscle masses of the rump of a deer by means of an air pistol.

The air pistol. As worked out, a .22 caliber Webley air pistol was modified by placing in the bore an 18″ piece of thin-walled brass tubing having an inside diameter of 0.17″ and an outside diameter sufficient to give a close, tight fit. This increased the length of the barrel and eliminated rifling which tended to break up the testosterone slugs. The long, slim barrel seemed to increase the velocity and penetration of the slug. Any air gun having a comparable bore and power, such as a Crossman rifle or pistol, could be adapted in this manner. The modified air pistol and the testosterone bullets which it shoots are shown in figure 1.

The testosterone slugs. Slugs were prepared of pure crystalline testosterone propionate (Roche-Organon, Inc.)[4] with a small amount of glucose as a binder, rolled into cylinders slightly less than 0.17″ in diameter and about $1\frac{3}{8}$″ in length, each containing 350 mg. of testosterone. These cylinders were

[3] The operative procedures were carried out mainly by Drs. John L. Langohr and Don W. Fawcett of the Harvard Medical School to whom we wish to express our appreciation

[4] Throughout this paper the word testosterone, when it refers to an injected drug, means testosterone propionate. Large amounts of this compound have been generously given to us through Mr. Marti of Roche-Organon, Inc. To them we are very grateful.

dusted with lycopodium spores, forced through a die to ensure correct size and were then shaped at one end into a conventional bullet nose (fig. 2).[5] These slugs were firm and tough enough to retain their shape with reasonably careful handling and showed little tendency to shatter or distort on impact. Materials were kept clean, but aseptic handling was not attempted since contamination was inevitable in passage through the deer's hide.

Procedure for shooting the slugs into the deer. The deer having been driven into a narrow runway constructed of wooden slats, the barrel of the pistol was poked between the slats with the muzzle touching the rump of the deer at a point slightly below the level of the external genitalia. The pistol barrel was directed as nearly as possible parallel to the axes of the hairs. The rump with its large muscle mass was chosen as the site into which to shoot the slugs

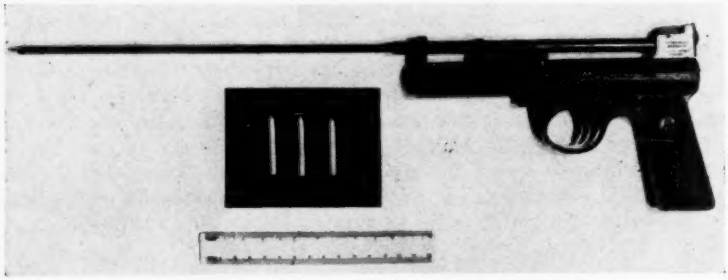


FIG. 1. A photograph of the air pistol and the testosterone slugs which it shoots.

so as to avoid possible injury to viscera and important blood vessels and nerves.

When the gun was aimed and the trigger pulled, the slug was released with sufficient force to pierce the hide and penetrate the muscles to a depth of one or two inches. Uniformly successful penetration could only be obtained by holding the barrel of the pistol almost in contact with the deer's coat. In this position, air preceding the slug parted the hair, maximum velocity was obtained, and the slug had little chance to turn. The thicker winter coat of the deer offered more resistance to penetration of the bullets than the summer coat, and here we had the occasional experience of a slug being deflected or going only part way in and breaking off. These differences in summer and winter conditions will be met fully, we anticipate, by a further modification in the design of the gun on which we are now at work.

Reaction of the animal to the shots was surprisingly mild and, in most instances, the only result of a shot was a slight jerk on the part of the deer accompanied by the appearance of a drop or two of blood at the point of entry of the bullet.

The inconspicuous wounds healed completely within a few days. In one animal which was autopsied a month following the introduction of three bullets, dissection revealed the slugs broken into a number of pieces and

[5] Advice and assistance in preparation of the slugs were given by Dr. L. M. Ohmart of the Massachusetts College of Pharmacy to whom we wish to acknowledge our indebtedness.

located an inch or two beneath the skin. There was no evidence of infection in the region, nor was there any damage of consequence to the tissues.

SEASONAL CHANGES IN ANTLERS AND GONADS IN NORMAL VIRGINIA DEER

It is unnecessary to review here in detail the morphological changes which the deciduous antlers of deer undergo annually or to present the related seasonal changes which occur in the male reproductive tract. Detailed accounts of these changes will be found in previous papers (Wislocki, '42, '43).

Briefly, it may be said of the Virginia deer that, as in other members of the Cervidae, the antlers are deciduous, being annually renewed. In the temperate zone of the northern hemisphere the antlers of the Virginia deer (like those of most other deer) begin to grow in April or May; they continue to mature and to become fully calcified by late summer, shedding the velvet in September. Rut ensues in October and November and the bare antlers are cast off in mid-winter. With variations of several months in the calendar, this sequence of events is essentially the same in all North American Cervidae (Caton, 1877; Seton, '09; Noback and Modell, '30).

The Virginia deer also exhibits seasonal changes in the size and histological pictures of the testes and seminal vesicles. Examination of the testes of Virginia deer in North America reveals the presence of spermatozoa beginning in July, becoming maximal by October and diminishing by December and January. In April and May, the testes are in a state of maximal regression. The seminal vesicles, inactive in spring and early summer, commence to grow in midsummer and reach a high degree of activity by October.

The antlers begin their annual renewal at a time when the testes and seminal vesicles are most inactive; they become calcified and the velvet is shed when the testes and seminal vesicles are rapidly enlarging, while the antlers are lost in mid-winter when the testes are declining. These relationships are presented in graph 1.

EXPERIMENTAL RESULTS

The present report brings up to date a series of experiments conducted since 1942, but it does not conclude the experiments. A number of the experimental animals are still alive and others have been recently prepared so that we anticipate continuing our investigations.

The present report concerns the following topics: (1) the influence of castration of deer at different ages (fawns and adults); (2) the influence of administered testosterone propionate on antler growth in castrated deer; (3) the effects of administration of testosterone on normal bucks; and (4) the induction of antler growth in ovariectomized does by administration of testosterone propionate.

The influence of castration, during the first year of life, on the growth of antlers. Three deer were castrated as fawns, two of them on Sept. 22, 1943, at approximately 4 months of age (no. 110 and no. 114), and the third one on Jan. 19, 1944, at 8 months of age (no. 156). The testes of each deer at time of removal weighed 10, 11 and 8.5 gms. respectively. No. 156 showed absolutely no sign of growth of either antler-

pedicles or antlers in the two following summers. Nos. 110 and 114 developed slight bony nubbins beneath the skin of the forehead (antler pedicle primordia) in the months following operation, but no antler growth whatsoever by the subsequent spring and summer.

These three experiments indicate that deer castrated as fawns do not develop antlers.

The influence of castration of adult deer on antler growth. This topic was studied on three adult Virginia deer, two of which (nos. 221 and 204) we castrated ourselves and one of which, already castrated, was presented to us by Dr. H. L. Ratcliffe of the Philadelphia Zoological Garden. The latter we will refer to henceforth as the "Philadelphia deer."

No. 221 was castrated at 2 years of age at a time of the year (on June 8, 1943) when the antlers were in the velvet and 2 inches long. The animal weighed 120 lbs. and the removed testes weighed 24 gms. Subsequent to operation, the antlers of this animal remained permanently in the velvet and were not shed until induced to do so later by the administration of testosterone. On Nov. 17, the antlers were still in the velvet. There were no signs of rutting behavior and the neck was not swollen as in normal males at this period. In December, the velvet began to freeze back from the tips of the antlers exposing the calcified tines beneath. The tip of the left antler broke off and dangled by a piece of shrivelled velvet. This fragment was cut off and examined; it was $9\frac{1}{2}$ cm. long and was covered by torn, desiccated velvet; it was noticeably poorly calcified.

In the course of the winter, the velvet froze back more and more toward the base of the antlers. Furthermore, in January the partially denuded frozen extremities of the antlers broke off on each side within an inch and a half of their pedicles, leaving persisting antler stumps covered, except for their broken ends, with viable vascularized velvet. Soon thereafter hairless skin regenerated over the stumps at the sites of amputation. By June, 1944, fresh antler tissue had sprouted in fairly normal fashion from the abnormal persisting stumps of the previous winter. By July, fairly normal and sizable antlers had grown out from the old stumps of last year.

The second castrated adult, no. 204, differed from the first one in particular by having been castrated in November, at a time when the antlers were normally denuded of velvet, instead of in June. No.

PLATE 1

FIG. 2A. A Virginia deer nine years of age which was castrated as a yearling. Following castration, the antlers remained permanently in the velvet, were not shed, and continued to grow irregularly with the result shown here (winter 1946). Shortly after this photograph was made, testosterone was administered (Mar. 15, 1946), and two months later the antlers were shed (May 15–20, 1946).

FIG. 2B. The same animal on July 27, 1946, showing new antler growth. This photograph, and that shown in figure 2A, were obtained through the kindness of Dr. H. L. Ratcliffe of the Philadelphia Zoological Garden.

PLATE 1

204 was castrated on Nov. 19, 1942, at the age of 17 months (fig. 3). Its testes, being in the season of greatest sexual activity, weighed 66 gms. (in contrast to those of no. 221 removed in June which weighed only 24 gms.).

Within two weeks' time ($12\frac{1}{2}$ days), the antlers of this deer were prematurely cast off; they were found in the paddock on the morning of Dec. 2. It should be pointed out that normal shedding of the antlers by a deer of this age would not have been anticipated before January or February. This animal commenced to grow a new set of antlers at the regular time the following spring. By June 8, 1943, the antlers were reported as growing; they were about 1 inch long and appeared to be slightly deformed. The right antler became tripartite and suffered bruises, besides being fractured and losing several pieces from its tips during the course of the summer (fig. 4). By November, 1943, the antlers were still in the velvet and showed no further fractures or loss of tines. The animal was in fine shape, but, like no. 221, it showed no aggressive behavior and no swelling of the neck characteristic of rut. Instead, both deer were exceedingly docile and gentle. By December, 1943, the velvet began to freeze back from the tips, exposing calcified portions of the tines. Late in January, the denuded, frozen antlers broke off $1\frac{1}{2}$ inches above the burr or corona, leaving abnormal antler stumps attached to the pedicle. These stumps remained covered by their original velvet and were vascularized. Soon thereafter the velvet surrounding these stumps grew out over the distal surface of each stump to form a complete layer of protective skin. By June 1, 1944, new antler buds had appeared on the stumps of last year's antlers. The right bud was quite smooth and normal looking; the left bud appeared to have been bruised and lacerated. A small globoid piece became dislocated and was attached loosely by a vascularized pedicle to the main bud. This piece was lost a few days later.

The third adult castrated deer, which we have called the "Philadelphia deer," is nine years old and was castrated as a yearling. It developed very typical castrate antlers and since it was kept indoors in the winter and hence protected from frostbite, it has accumulated an excess of antler tissue. As is typical of castrate deer, the antlers have never been shed, but have remained, instead, permanently in

---

PLATE 2

FIG. 3. Deer no. 204 at approximately 17 months of age (Nov. 1942) when it was castrated. Within 12 days following castration, the antlers seen in the picture were shed quite suddenly and prematurely.

FIG. 4. Deer no. 204 in August of the following year (1943), showing the antlers which renewed themselves in spite of castration. These antlers remained permanently in the velvet and were not dropped until a year later when the animal was given testosterone.

FIG. 5. Susan, an ovariectomized deer which developed antlers, as shown in the photograph, following the administration of testosterone.

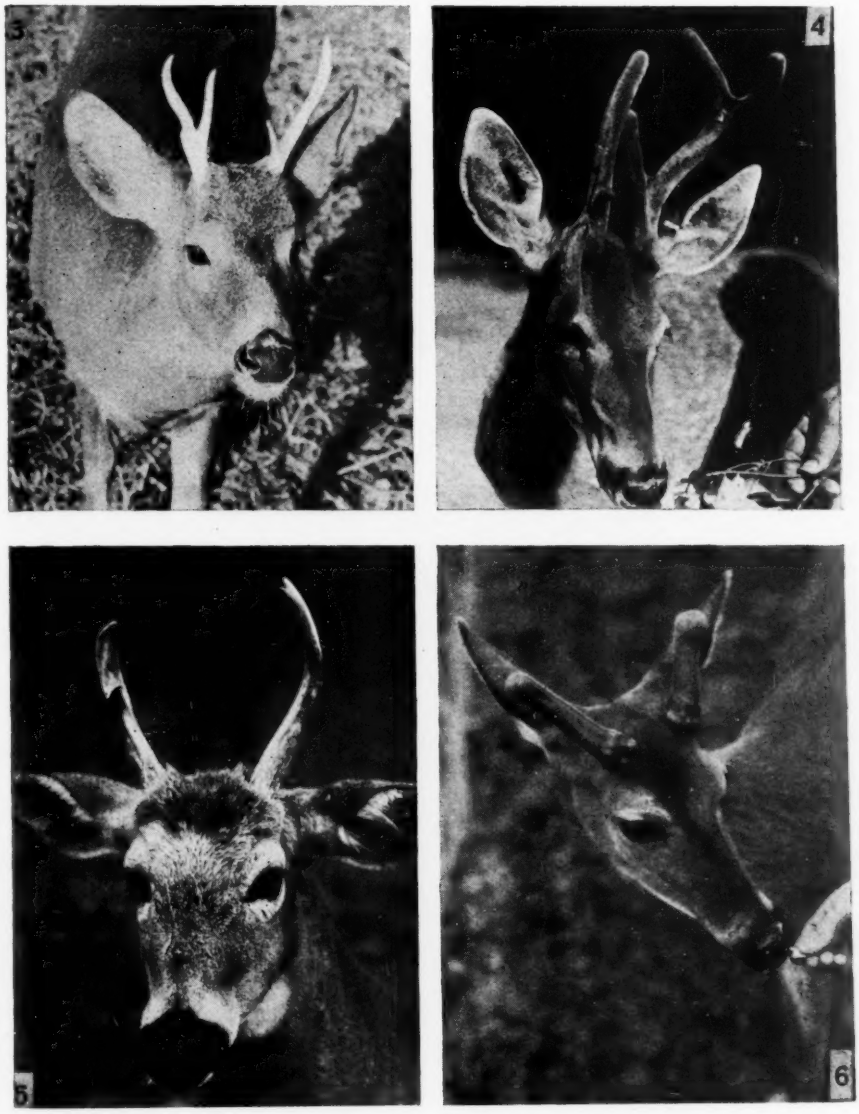


PLATE 2

FIG. 6. Deer no. 110 castrated at 4 months of age (Sept. 1943). Testosterone was given in July 1944, followed by the appearance of antler pedicles during 1944 and a good set of antlers in 1945. These "castrate" antlers, permanently in velvet, were reduced by freezing to stubs about an inch high by spring 1946. The photograph presented here shows these persistent stumps surmounted by additional growth in June 1946. It will be observed that growth has taken place from the tips of the stumps instead of from the margins of the original antler pedicles.

PLATE 3

FIG. 7. Deer no. 110, castrated at 4 months of age, showed no antler growth whatsoever until testosterone was administered. The picture, taken on July 29, 1944, 31 days following the administration of testosterone, shows the prominent pedicles, 1 inch long, where none had been before.

FIG. 8. Two castrate deer, both of whom had shed their castrate antlers in the previous year following injections of testosterone. They are shown on July 31, 1945. No. 221, with antlers, had had nothing done to it in the preceding spring, whereas no. 204, without antlers, had received 950 mg. of testosterone in March, 1945. This dose of testosterone inhibited the regrowth of antlers until August, when delayed castrate antlers reappeared.

FIG. 9. No. 204 of the preceding picture, in October 1945 when somewhat deformed

the velvet as slow-growing, vascularized, semicalcified, deformed antlers of bizarre proportions (fig. 2 A).

These three animals illustrate the influence on antler growth of removal of the testes from adult Virginia deer. If castration is carried out during the period when the antlers are growing, the velvet is never shed and the antlers are never cast off. If castration is performed at a season when the antlers are denuded of velvet and dead, the antlers are prematurely shed within one or two weeks; but the subsequent year they are renewed and thereafter remain permanently in the velvet continuing to grow by annual increments. This process is modified in so far as in winter the antlers become frost bitten and variably destroyed. In animals which are adequately protected by indoor shelters or live in milder climates, this denudation of the velvet and destruction of the antlers by freezing are minimal and the castrate antlers become correspondingly large. The effects of castration, as exemplified in our experiments on adult deer, are illustrated in graph 1. Thus, our results in adult deer differ from those obtained in fawns, which develop no antlers whatsoever after castration.

The influence of testosterone on antler growth in castrated deer. We have demonstrated in the previous section that there are very distinct prepuberal and postpuberal reactions of the antlers to castration. Removal of the testes in the first year of life suppresses antler growth completely, whereas their removal at any time after the first pair of antlers has begun to grow results in eventual permanent retention of the antlers in the velvet. In view of these differences, one would anticipate differences in the effects exerted by testosterone in deer castrated before and after puberty. This proves to be the case.

No. 110, castrated when 4 months old (Sept. 1943), was given 1050 mg. of testosterone on June 28, 1944 up to which time there had been no signs of antler growth. During the first weeks of July this animal showed marked sexual excitement. Antler pedicles began to make their appearance (fig. 7), and by October the animal had fully formed antler pedicles surmounted by small, naked bony tips which were $\frac{1}{2}$ centimeters long. By early spring, the bony tips had disappeared and the ends of the pedicles were covered over by shiny, hairless skin, resembling the pedicles of normal animals at that season. In May, without any further priming by testosterone, antlers began to grow and by fall, the animal had a good set of antlers some 10″ long, exhibiting 4 points on the left side and 2 on the right. As in adult castrate deer, these antlers now remained in the velvet (Nov. 20,

---

castrate antlers had reappeared. Being a castrated buck, these antlers should remain in the velvet and will not be shed unless testosterone is again administered.

FIG. 10. Castrate deer no. 221, showing the 1945 antlers in the velvet in October, after losing its castrate antlers in the previous year following administration of testosterone. The antlers seen in the picture are, at present, still in the velvet (April, 1946), although the tines have been greatly reduced by frostbite.

1945) and during the winter became frozen and reduced to stumps about 1 inch long. Subsequently in the following spring the antlers were renewed by outgrowth of tissue from these residual stumps. The appearance of the antlers on June 13 is shown in figure 6. As described in previous passages, a similar appearance of the antlers was seen in deer 221 and 204.

No. 114, castrated when 4 months of age (Sept. 1943), was given 1050 mg. of testosterone on June 28, 1944, up to which time no antler growth had become apparent. After receiving testosterone, this animal became uneasy and much wilder than previous to the injection. As a result of this, the animal accidentally killed itself one month later (July 29). At the time of death, distinct growing antlers, about $\frac{3}{4}$ inches in length, had become apparent. Examined histologically, these nubbins revealed, at their tips, proliferating tissue characteristic of normally growing antler pedicles.

No. 156, castrated when 8 months old (Jan. 19, 1943), grew no antlers whatsoever in the following three summers (1943-44-45). On July 13, 1945, this deer was given in the neighborhood of 500 mg. of testosterone. After receiving testosterone, the animal became bumptious, but no growth of antler pedicles was observed by fall. Small nubbins "about the size of a thumbnail" were seen on this animal's forehead in December, but in January, they appeared to recede. On April 5, 1946, there was no sign of antler growth but, by May 27th, a right antler had begun to grow and had formed a spike about 3 inches long. There was, at this time, no evidence of growth of a left antler. However, on June 6th, it was reported that a left antler was just visible and on June 13th, that it was $\frac{3}{4}$ of an inch long; the right antler, meanwhile, had become an unbranched spike 5 inches in length. The growth of antlers in these three castrates illustrates that the "priming" with testosterone of the year before was sufficient to invoke spontaneous growth in the ensuing year.

The three preceding experiments represent our experience with prepuberal male castrates. The three following experiments set forth our experiences with testosterone administered to post-puberal castrate bucks.

No. 221. This deer was castrated on June 8, 1943, at 2 years of age and thereafter retained its antlers permanently in the velvet and without shedding them. On June 29, 1944, it was given 1050 mg. of testosterone. Following this injection, the antlers became completely denuded of velvet by Aug. 23. The loss of velvet extended all the way down to the permanent antler pedicles. It cast off its left antler on Dec. 17, 1944, and the right one on the following day.

It is apparent that the antlers in this animal became denuded of their velvet a short while following the injection of testosterone and were shed some months later. From our knowledge of the antlers in castrate deer, this would not have happened without the injection of testosterone.

This animal was allowed to proceed through the year (1945) without any further experimentation. In April, it began to develop a set of new antlers, and by May, it had nicely forked antlers which were 5″ or 6″ long. In August, it had very handsome 14″ antlers with 8 points (fig. 8), and in November, these antlers were still intact and carrying their velvet (fig. 10). As occurs invariably under the conditions of our experiments, the tips of the antlers became frostbitten in January, 1946, and the frozen parts broke off, about 3 inches of the original antlers remaining on the head by the beginning of spring.

No. 204 was castrated at the age of 17 months (Nov. 9, 1942) and shed its antlers within two weeks. It developed antlers the following May which remained permanently in the velvet through the following winter and spring (1944), although they were considerably reduced in size by frostbite. On June 1, 1944, new antlers were sprouting on the old stumps of the previous year's antlers. On June 28, 1944, this castrated animal received pellets of testosterone amounting to 1050 mg. A month later (July 29), the right antler had become completely denuded of velvet, while the left antler showed desiccated velvet beginning to come off in shreds. This shedding included the velvet covering the surface of the old stumps right down to the pedicle. Through July and August, the animal showed sexual excitement with repeated erections of the penis. The completely denuded antlers were carried until Nov. 3, when the right antler was shed and, on Nov. 4, the left antler toppled off.

It is apparent in this animal that, as a consequence of receiving testosterone, the velvet was promptly shed followed, some months later, by the dropping off of the denuded antlers.

We have data obtained from deer no. 114, which died accidentally 30 days after administration of 1050 mg. of testosterone, indicating that 347 mg. had been resorbed or approximately 11 mg. per day. Hence, 1050 mg. should require about 3 months for complete absorption. From these considerations, it becomes apparent in deer no. 221 and 204 that, whereas the velvet was shed at the height of testosterone absorption (one month after injection), the desiccated antlers were cast off at a time when the testosterone was greatly diminished or had been completely used up (4 months after injection).

It should have been anticipated that no. 204 would have developed a new set of antlers the following spring just as deer no. 221 did. Instead of waiting to observe this outcome, no. 204 was given between 900 and 950 mg. of testosterone on March 16, 1945, at a time when no new antlers had yet appeared. This unusual interference was followed by no antler growth whatsoever, but instead by an actual desiccation of the delicate skin covering the pedicles from which any new growth would have had to take place (fig. 8). Small bare patches of bone appeared where the skin or velvet had disappeared. Thus, the skin covering the pedicles at this season behaved towards testosterone much as the velvet of the growing antler does later in the season.

1945) and during the winter became frozen and reduced to stumps about 1 inch long. Subsequently in the following spring the antlers were renewed by outgrowth of tissue from these residual stumps. The appearance of the antlers on June 13 is shown in figure 6. As described in previous passages, a similar appearance of the antlers was seen in deer 221 and 204.

No. 114, castrated when 4 months of age (Sept. 1943), was given 1050 mg. of testosterone on June 28, 1944, up to which time no antler growth had become apparent. After receiving testosterone, this animal became uneasy and much wilder than previous to the injection. As a result of this, the animal accidentally killed itself one month later (July 29). At the time of death, distinct growing antlers, about $\frac{3}{4}$ inches in length, had become apparent. Examined histologically, these nubbins revealed, at their tips, proliferating tissue characteristic of normally growing antler pedicles.

No. 156, castrated when 8 months old (Jan. 19, 1943), grew no antlers whatsoever in the following three summers (1943-44-45). On July 13, 1945, this deer was given in the neighborhood of 500 mg. of testosterone. After receiving testosterone, the animal became bumptious, but no growth of antler pedicles was observed by fall. Small nubbins "about the size of a thumbnail" were seen on this animal's forehead in December, but in January, they appeared to recede. On April 5, 1946, there was no sign of antler growth but, by May 27th, a right antler had begun to grow and had formed a spike about 3 inches long. There was, at this time, no evidence of growth of a left antler. However, on June 6th, it was reported that a left antler was just visible and on June 13th, that it was $\frac{3}{4}$ of an inch long; the right antler, meanwhile, had become an unbranched spike 5 inches in length. The growth of antlers in these three castrates illustrates that the "priming" with testosterone of the year before was sufficient to invoke spontaneous growth in the ensuing year.

The three preceding experiments represent our experience with prepuberal male castrates. The three following experiments set forth our experiences with testosterone administered to post-puberal castrate bucks.

No. 221. This deer was castrated on June 8, 1943, at 2 years of age and thereafter retained its antlers permanently in the velvet and without shedding them. On June 29, 1944, it was given 1050 mg. of testosterone. Following this injection, the antlers became completely denuded of velvet by Aug. 23. The loss of velvet extended all the way down to the permanent antler pedicles. It cast off its left antler on Dec. 17, 1944, and the right one on the following day.

It is apparent that the antlers in this animal became denuded of their velvet a short while following the injection of testosterone and were shed some months later. From our knowledge of the antlers in castrate deer, this would not have happened without the injection of testosterone.

This animal was allowed to proceed through the year (1945) without any further experimentation. In April, it began to develop a set of new antlers, and by May, it had nicely forked antlers which were 5″ or 6″ long. In August, it had very handsome 14″ antlers with 8 points (fig. 8), and in November, these antlers were still intact and carrying their velvet (fig. 10). As occurs invariably under the conditions of our experiments, the tips of the antlers became frostbitten in January, 1946, and the frozen parts broke off, about 3 inches of the original antlers remaining on the head by the beginning of spring.

No. 204 was castrated at the age of 17 months (Nov. 9, 1942) and shed its antlers within two weeks. It developed antlers the following May which remained permanently in the velvet through the following winter and spring (1944), although they were considerably reduced in size by frostbite. On June 1, 1944, new antlers were sprouting on the old stumps of the previous year's antlers. On June 28, 1944, this castrated animal received pellets of testosterone amounting to 1050 mg. A month later (July 29), the right antler had become completely denuded of velvet, while the left antler showed desiccated velvet beginning to come off in shreds. This shedding included the velvet covering the surface of the old stumps right down to the pedicle. Through July and August, the animal showed sexual excitement with repeated erections of the penis. The completely denuded antlers were carried until Nov. 3, when the right antler was shed and, on Nov. 4, the left antler toppled off.

It is apparent in this animal that, as a consequence of receiving testosterone, the velvet was promptly shed followed, some months later, by the dropping off of the denuded antlers.

We have data obtained from deer no. 114, which died accidentally 30 days after administration of 1050 mg. of testosterone, indicating that 347 mg. had been resorbed or approximately 11 mg. per day. Hence, 1050 mg. should require about 3 months for complete absorption. From these considerations, it becomes apparent in deer no. 221 and 204 that, whereas the velvet was shed at the height of testosterone absorption (one month after injection), the desiccated antlers were cast off at a time when the testosterone was greatly diminished or had been completely used up (4 months after injection).

It should have been anticipated that no. 204 would have developed a new set of antlers the following spring just as deer no. 221 did. Instead of waiting to observe this outcome, no. 204 was given between 900 and 950 mg. of testosterone on March 16, 1945, at a time when no new antlers had yet appeared. This unusual interference was followed by no antler growth whatsoever, but instead by an actual desiccation of the delicate skin covering the pedicles from which any new growth would have had to take place (fig. 8). Small bare patches of bone appeared where the skin or velvet had disappeared. Thus, the skin covering the pedicles at this season behaved towards testosterone much as the velvet of the growing antler does later in the season.

Following this suppression of the delicate germinal tissue of the antler pedicles, nothing further happened until August when it was noticed that the tissue at the tips of the antler pedicles was beginning to grow. It should be noted that this was some 4½ months after the previous administration of testosterone and hence by our calculation well after the effect of the testosterone had worn off. By November, 1945, this castrated animal had again developed small antlers which had retained their velvet beyond the time when a normal animal of similar age would have shed it (fig. 9). Observations on this animal were abruptly terminated on Nov. 20, when it was stolen.

The third adult castrated deer, it will be remembered, was called the "Philadelphia deer." This animal, which was presented to us through the generosity of Dr. H. L. Ratcliffe, had been castrated as a yearling and is now nine years old. Since it was sheltered in the winter against frostbite, it had luxuriant abnormal masses of antler tissue on its forehead which have remained perpetually in velvet (fig. 2A).

On March 15, 1946, this animal was given two pellets of testosterone (700 mg.). In the week following injection, the velvet covering the antlers seemed somewhat moister than usual, but in the following two weeks the velvet gradually became dryer, until by April 5, the antlers had become dry and cool and the velvet began to shed. Shedding continued throughout the next two weeks until the antlers were completely denuded. On April 2, and again on April 5, sexual activity (pelvic thrusts) was noticed. On May 15, sixty-one days after receiving testosterone, the right antler dropped off leaving a large, conical, bony stump, some 6 cm. in length. Blood oozed from this surface immediately after shedding and for several hours thereafter. On May 20, the remaining antler dropped off, leaving a bony stump similar to the one on the right. Within 17 days fresh velvet was beginning to appear and by July growth of bizarre new antler tissue had reached the state visible in figure 2B.

The experiments with testosterone on these three castrated adult deer illustrate several of its actions. Its administration to castrates causes the velvet to be shed promptly, thereby tending to restore the cyclical changes which the antlers of adult castrates invariably lose. Noteworthy also is that the dead antlers in these experimental animals do not actually drop off until the testosterone has been absorbed resembling, in this respect, the antlers of normal deer which are shed during a period of declining gonadal function (see graph 1).

Another influence of testosterone emerges from comparison of deer no. 221 and no. 204 in the spring and summer of 1945 when one was given testosterone in March and the other not. No. 221, which received no injection, developed a fine set of antlers, whereas antler growth was delayed in no. 204 until the effect of the testosterone injection had worn off. This experiment seems to indicate an inhibition of antler growth by testosterone in the adult animal due to premature shedding of the velvet over the antler pedicles.

The effects of administration of testosterone propionate to normal male deer. Our experiments on this topic have been rather inconclusive and, in the case of one experiment, contradictory, but they are, nevertheless, of some interest. We have administered testosterone to six normal male deer.

Two male yearlings (approximately 12 months old) were given testosterone on May 6, 1945. No. 175 received 500 mg., while no. 277 received 1150 mg. At the time of injection, the antlers had begun to grow, the buds being about ¾ inch tall. To our surprise, the antlers of these two animals did not behave in the same way. No. 175 grew essentially normal antlers during the summer which lost their velvet on Sept. 15. No. 277, on the contrary, had difficulty with its antlers from the time of injection on; the velvet covering the antler buds was promptly denuded, exposing bare bone, and no further growth of the antlers took place. We would have anticipated premature shedding of the velvet in both animals under the influence of testosterone at this stage of antler development. It is conceivable that 500 mg. of testosterone, which is what no. 175 received, is not a large enough dose to cause shedding of the velvet at the time when the antlers are beginning to grow and when the animal's own testes are in a state of complete regression.

Another yearling deer, no. 280, received 250 mg. of testosterone on July 13, 1945, at a time when its antlers were two inches long. Following this relatively small dose of testosterone the animal's antlers continued to grow, although they were rather small, malformed and spindly. Nevertheless, the velvet did not desiccate completely until two months later. The small, malformed antlers seemed poorly calcified for one of them broke off around the first of October leaving only a small stump behind. In January, 1946, this animal still carried a three-inch spike on the right and a one-inch ragged stump on the left; these were shed on Feb. 4 and 8.

One may conclude from these three observations that in so far as testosterone influences the growing antlers of yearling deer, it leads to injury of the velvet and variably severe interruptions of growth.

Testosterone was also administered to a vigorous, normal six year old buck named "Ichabod." On July 29, 1944, when this animal had a full set of antlers in the velvet, he was given 1050 mg. of testosterone. In the days and weeks immediately following the injection, he became aggressive, hyperactive and dangerous, his behavior being that of the normal period of rut. By August 26, within less than four weeks after injection of testosterone, the velvet covering his antlers had become desiccated and had been shed. The denuded antlers were subsequently carried for the normal period, being dropped on February 9, and 12, 1945.

On March 16, 1945, just as his new antlers were beginning to grow, "Ichabod" was again given between 700 and 1000 mg. of testosterone. For about two months thereafter, he became aggressive,

dangerous and hyperactive again. In the weeks following the injection, he knocked and bruised his growing antlers considerably. The antlers which continued to grow were very abnormal and somewhat stunted compared to the sets of the previous years; yet they retained their velvet and did not lose it until the end of September. The denuded antlers were eventually shed in mid-winter, the right on December 28, and the left on January 1, 1946.

In addition to the preceding four animals, testosterone was also given to deer no. 169 under the following circumstances. This deer, a vigorous adult of unknown birth date, had well-developed growing antlers about 6 inches long in June, 1944. On June 28, 1944, this animal was anesthetized with nembutal and about an inch of the tip of the growing right antler was removed surgically in order to implant small bits of the proliferating osteogenic tissue in various subcutaneous locations in the body of the animal. Before completing the operation, 700 mg. of testosterone were implanted in the rump of the animal. Within a few days, the amputation stump, where the small piece of tip had been removed from the right antler, appeared to have healed completely and the antler seemed to have resumed its normal growth. By the end of July, the velvet on both of the antlers had begun to wither. Meanwhile, on July 29, one more pellet of 350 mg. of testosterone was introduced into the animal. By August 22, the antlers had become completely denuded, a matter of weeks earlier than was to be expected. The antlers were shed eventually at approximately the normal time on January 21, 1945. It may be added, for completeness' sake, that none of the seven small subcutaneous transplants of antler tissue survived or grew.

Testosterone has also been given to two other animals, one a normal buck and the other a thyroidectomized male, to test a concept advanced in a previous article (Wislocki, '43) that the dead antlers are shed during a period of very much diminished gonadal function. If this be true, it would seem reasonable to expect that administration of testosterone at that period would delay or indefinitely postpone shedding of the antlers.

With this in mind, seven year old Bobby, no. 74, was given 500 to 700 mg. of testosterone on January 30, 1946, before he had shed either of his well-developed, normal antlers. On April 5, Bobby still carried both of his antlers, and so as to continue the experiment, he was given an additional 700 mg. of testosterone. Bobby carried his antlers, after this second injection, for six additional weeks until May 18 and 19, when first the right antler and then the left was shed. It was observed that the pedicles remaining behind were larger in diameter and that the skin at the circumferential margins was thicker and more conspicuous than usual. The outcome of this experiment bears out our concept that the normal shedding of the antlers is dependent on a diminishing activity of the testes. It is of interest, also, that the ab-

normally persisting antlers prevented the growth of new antlers, which normally should have been two to three inches long by May 20. Following the delayed shedding of Bobby's antlers, new ones began to grow at once, but, growing from an unusually broad base, they were exceptionally thick, and, since they did not commence to grow until relatively late, they had attained a length of only two inches by June 13. When the velvet was shed on Sept. 18, the antlers were over twelve inches long, but their tips were still blunt, indicating that growth was never completed.

No. 174, a yearling which possessed a good set of antlers, was also given 1050 mg. of testosterone on January 30, 1946. It should be remarked of this animal that it had its thyroid glands removed at two months of age (July, 1944) a procedure which we have reason to believe also retards shedding of the antlers. Thus, there were two separate reasons for expecting that shedding of the antlers of this deer would be postponed. This animal was not given any subsequent injections of testosterone. It finally shed both of its antlers on May 9, some two and a half months later than a normal animal should have been expected to lose them. Following loss of the old ones, new antlers were slow in appearing and, as reported on June 13, the new ones were no more than $\frac{3}{4}$ inch high.

The induction of antler growth in does by administration of testosterone propionate. With the exception of females of reindeer and caribou which regularly grow antlers, the does of other species of the Cervidae develop antlers only very rarely. We have been successful in inducing the growth of antlers in two ovariectomized white-tailed deer by the administration of testosterone.

One of these two animals was "Susan", a doe who was born in 1938, and who had produced young on several occasions. In January, 1944, this animal was ovariectomized, including a complete hysterectomy, by Glenn Morton and E. L. Cheatum. Susan had a peculiarity, occasionally encountered in female deer, that she had small protuberances which could be palpated beneath the skin on the forehead where the antlers are located in males. The presence of these anomalous exostoses led us to give Susan some testosterone. On June 29, 1944, she received 350 mg. of testosterone. On August 22, 1944, it was reported that there was no visible change in the buttons on Susan's forehead. However, it was observed in October that the bony exostoses on Susan's head had become slightly broader. Moreover, the hair over the nubbins appeared to have become coarser and longer. Yet, shortly thereafter, the nubbins began to diminish and by the end of December, they had returned again to their original size. In view of the above observations, Susan was given 1050 mg. of testosterone on March 16, 1945. Late in April, it was recorded that there was definite enlargement again of the nubbins on Susan's forehead, and by June, they had become definite antlers, the one on the right protruding

about 2 inches, while the left one was 3 inches long. By July, they were 5 and 4 inches long respectively, and both were bifurcated.

On July 13, 1945, she was given further testosterone to the amount of 700 mg. The antlers continued to grow, reaching a maximum length of 6½ inches on the right and 8 inches on the left, and the velvet was shed in the first week of September. The dead antlers were carried on the forehead through the fall (fig. 5) and winter until they were shed on Jan. 8, 1946. As in males, two definite pedicles remained on the forehead.

The antlers which were shed proved to be not unlike well-developed ones seen on a yearling buck except for one very definite difference. This peculiarity involves the base of the antlers which, in the case of Susan, have practically no burr or corona as well as relatively low and inconspicuous longitudinal ridges. Furthermore, and unlike normal male antlers, the bone at the proximal ends of the shed antlers, instead of being convex, presents a perfectly flat surface as though it had been separated from the pedicle by a straight knife cut. These peculiarities, especially the absence of burr and longitudinal ridges, are said to be characteristic of the antlers which occur now and then spontaneously in females of those species of Cervidae in which the female does not ordinarily bear antlers (cf. Rörig, '07).

Our second case of induced antler formation in a female deer is not unlike Susan in her first year. No. 133 had both ovaries removed on January 19, 1943, at about 8 months of age. Over two years later, on May 5, 1945, she was given 1050 mg. of testosterone. Unlike Susan, the preceding doe, this animal had not the slightest indication on her forehead of any bony elevations. By June 11, this doe had developed two prominent bony nubbins palpable underneath the skin of the forehead and each about ½ inch long. By October, these exostoses had become perhaps ¾ of an inch long. At all times, they had hair and not velvet covering them. By November, these nubbins had receded, but even in late winter (Feb. 1946), they could still be palpated on the forehead. This condition was maintained until after May 21, 1946, when growth was renewed, the nubbins having reached a length of ¾ of an inch by June 13, 1946. By Sept. 19 the antlers were still in velvet, the right one having reached a length of two inches, that on the left, one inch.

DISCUSSION

The hormonal control of antler growth. For reasons presented elsewhere (Wislocki, '43), it seems reasonable to assume that the gonadal and antler cycles in deer must be mainly under the control of the anterior pituitary gland and the testes. The data from normal deer, as well as observations on castrated ones, indicate that the hormones of these glands balance and supplement one another in controlling the growth, shedding and renewal of the antlers. The observations

made in the present experiments, on the growth of the antlers in normal and castrated deer receiving testosterone, provide additional evidence supporting such a hypothesis.

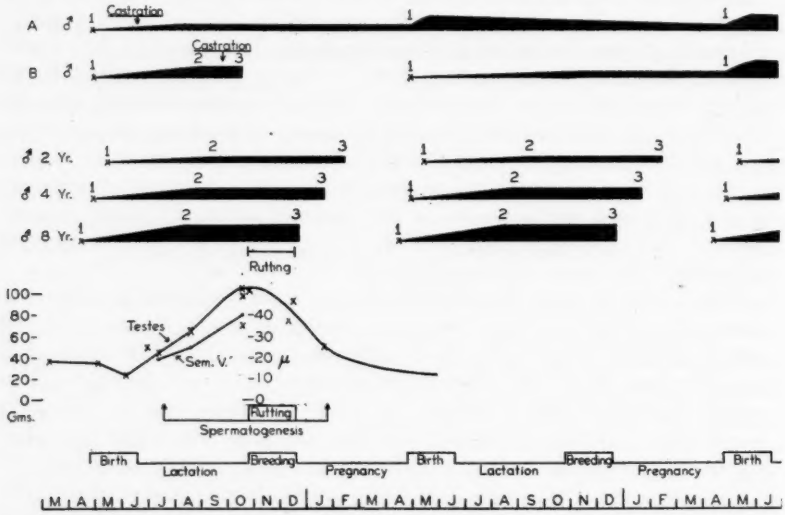
The permanent retention and continuing growth of the antlers following castration of adult deer would seem to indicate the presence in the body of an antler-growth promoting factor very probably emanating from the anterior pituitary gland. Yet, the fact that antler growth does not ensue when a deer is castrated as a fawn until, and unless, testosterone is administered on one occasion, illustrates the need for an initial "priming" of the pituitary or antler primordium by the gonad. Having received this initial stimulation, which in the intact animal occurs in the prepuberal period preceding the appearance of the first, or yearling, antlers, the anterior pituitary gland thenceforth is capable, apparently, of secreting antler-growth promoting hormone independently of the testes.

The various lines of evidence from study of normal deer and castrates, as well as those receiving testosterone, indicate that the anterior pituitary plays its role in the renewal and growth of the antlers, whereas the testes exert their influence by promoting the maturation and secondary calcification of the antlers besides bringing about the shedding of the velvet. The evidence for suggesting that the testes induce secondary calcification rests upon the observed difference between the poorly calcified antlers of castrated deer and the fully hardened antlers of normal deer. The evidence for the conclusion that testosterone is responsible for the shedding of the velvet derives from the demonstration that the injection of testosterone into adult castrated deer causes prompt shedding of the velvet, that its injection into normal deer at certain times leads to shedding of the velvet and, finally, that in the seasonal cycle of normal antlers, the velvet is shed at a time of rising function of the testes.

From the observed relationships of the antler and gonadal cycles, one must conclude that the maximal influence of the pituitary (spring and early summer) precedes the maximal influence of the testes (fall) by a number of months, so that as the pituitary influence is declining (late summer), the testicular stimulus is rising. In this predicated cycle, the pituitary gland presumably stimulates antler growth (late spring) and testicular growth (early summer), whereas the rising testosterone inhibits pituitary function (late summer and fall). Some of the relationships between antlers and gonads which are discussed here are illustrated diagrammatically in graph 1.

The shedding or casting off of the dead antlers takes place, as a rule, in mid-winter at a period when the testes are regressing and the pituitary, according to our concept, is beginning to be released from testicular inhibition. The role of the testes in antler shedding is clearly demonstrated in two of our experimental procedures. First, when a buck is castrated in the fall, the antlers drop off prematurely within

a week or two following removal of the testes. Contrariwise, when testosterone is administered to deer shortly before the anticipated time of shedding of the antlers, shedding of the horns is definitely postponed. In addition to the demonstrated role of the testes in antler



GRAPH 1, illustrating the periodicity of the antlers, testes, seminal vesicles, and breeding season of the Virginia deer. On the abscissa the months of the year are indicated, beginning with March. The curve for testes is derived from their monthly weights, their individual weights being designated by crosses. The curve for seminal vesicles is expressed in terms of microns (μ) representing the height of the glandular epithelium in successive months.

The periodicity of the antlers is schematically presented for 2-, 4-, and 8-year-old animals. Of the numerals appearing in the graphs (1) represents the time of onset of antler growth; (2) the completion of antler growth and the shedding of the velvet; while (3) shows the time of dropping of the antler.

At the top of the graph, the results of castration are shown. A, an animal in which castration was performed in June; B, in which castration took place in October. In the first case, A, the antlers remain in the velvet and are not shed thereafter, whereas in B, the antlers are prematurely shed and then permanently renewed in the following season. These two diagrams correspond to the courses of deer nos. 221 and 204. Observe that in the castrates there is a new increment of antler tissue each spring. Reprinted with permission of the University of California Press.

shedding, there is very probably also a pituitary component represented by the proliferative changes of repair and preparation for the subsequent renewal of the antlers, which are already manifest to some slight degree in the zone of amputation at the time of shedding.

Because the pituitary principle which promotes antler growth activates them year after year, it may not be identical with the ordinary pituitary growth hormone which is responsible for prepuberal body growth, including skeleton, of young animals. Moreover,

it cannot be equated with the gonadotrophic hormone because the annual period of activation of the deer's testes follows by some months the period of maximal antler growth. The activation of the antlers is somewhat analogous to the activation of the mammary glands by the lactogenic hormone, so that the principle which promotes antler growth might represent the male counterpart of the lactogenic hormone. It may be recalled that lactogenic hormone has been recovered from various male animals, although no targets for it have heretofore been recognized for it in male organisms.

The role of testosterone in the formation of the antlers is analogous, in some respects, to the role which it plays in the growth of the skeleton in general, with one major difference, namely, that skeletal growth is essentially completed soon after puberty, whereas antler growth repeats itself annually throughout life. Nevertheless, as in the case of growing bone, so, too, in the growth of normal antlers, testicular function is necessary for the completion of growth and calcification. And, just as in castrated, growing animals, the epiphyses do not close and the skeleton fails to differentiate fully, so, too, in castrated deer, calcification and differentiation of the antlers remain incomplete. Finally, just as administration of testosterone to castrates will accelerate epiphyseal closure and complete bone-growth, so, too, it tends very definitely, as we have shown, to repair the growth defects in the case of the antler.

Previous experimental work on antler growth and its hormonal control. Since the time of Aristotle, occasional deer of a variety of species have been castrated and the effects upon antler growth observed. A compilation of much of that literature may be found in articles by Rörig (1899, '07). Reindeer have been partially or completely castrated by the natives of Lapland for hundreds of years. Castrations to obtain documented observations have been carried out previously by Caton (1877) on Virginia deer and elk, and by Tandler ('10) and Tandler and Grosz ('13) on the European roe-deer and reindeer. In addition to the Virginia deer castrated by Caton, Townsend and Smith ('33) show several pictures of two Virginia deer which had been castrated and developed deformities of the antlers.

Caton (1877) was unable to explain the observed changes after castration, whereas Tandler and Grosz ('13) attempted to relate them to principles in the testes which regulate secondary sex characters. Olt ('27), speculating on the gonad-antler relationship based on considerations of castrated roe deer, concluded that testicular hormones are given off constantly and that there are four of them which regulate antler growth: one, which initiates antler growth; a second, which regulates growth; a third, which initiates resorption of the velvet by a marked local "eosinophilia"; and a fourth, which acts on the musculature of the blood vessels of the antlers inducing a nutritional disturbance so that the velvet dies and the antlers are eventually cast off.

In a second article, Olt ('28) concludes that the withering of the velvet and subsequent death of the antlers are due to seasonal inhibition of antler growth by the testes.

In contrast to Olt's concept of the nature of testicular hormones, our own observations indicate (1) that one testis hormone (testosterone) is accountable for the effects which the testes exert upon antler growth; (2) that testosterone is responsible for the final maturation and hardening of the antlers as well as for the shedding of the velvet, but is not responsible for the initiation of antler growth and (3) that, in contrast to maturation and shedding of velvet which occur on rising testicular function, the eventual shedding of the dead antlers occurs normally with declining testicular function or experimentally after removal of the testes by castration.

More recently, Blauel ('35, '36) castrated two roe deer and subsequently treated them with steroid hormones. The first of these two animals, castrated as a fawn, developed no antlers whatsoever and did not respond, according to Blauel, to a testis implant or to a single injection of testosterone. The second of these animals, castrated after puberty, developed typical castrate horns (so-called wig antlers in the roe deer). This animal was given follicular hormone ("progynon") on successive occasions which induced the complete shedding of the velvet within several weeks, followed by casting off of the antlers 8 weeks later. Two weeks following loss of the antlers, new antlers began to grow; soon thereafter estrogen was repeatedly injected for over a month and, as before, the velvet was shed, followed again by loss of the antlers some 7 weeks later. Blauel concluded from this experiment that administration of estrogen was responsible for the shedding of the velvet as well as for the subsequent loss of the antlers; briefly, according to him, estrogen caused the wig antlers to die and to be cast prematurely.

It is of interest that estrogenic hormone has the same effect upon the antlers of a castrated deer as testosterone propionate. Both induce shedding of the velvet. Yet we do not share Blauel's belief in the positive action of "progynon" in causing the antlers to be shed; in his case, as in our experiments, because of the long interval which elapsed, we regard withdrawal of the steroidal influence as being responsible for the shedding of the antlers.

Blauel is the first writer on this topic, to our knowledge, to suggest an involvement of the hypophysis in the regulation of gonadal-antler periodicity, although he advances no specific suggestion as to its probable role. Interesting is the fact that in Blauel's experiment on a castrated roebuck, the antlers renewed themselves almost immediately following induced shedding. In the Virginia deer, under similar conditions, renewal of the antlers awaits the usual season for growth to take place and does not occur prematurely as described for the roebuck. Rörig ('07) cites other similar cases in the roebuck where upon the loss of the dead antlers. following castration in the fall, the

castrate antlers begin to renew themselves almost at once. This peculiarity of the robuck indicates, in our estimation, an unusually strong potency of the anterior pituitary gland for this season of the year. Other species resemble the Virginia deer in this matter.

It is interesting that Blauel achieved essentially the same results by administering estrogenic hormones as we have with testosterone. The results with these two hormones suggest the possibility of experimenting with other steroid hormones, more particularly with progesterone, and testing them in relation to the induction of antler growth in both does and bucks.

The induction of antler growth in female deer. In connection with our successful induction of antler growth in does by administration of testosterone, it is interesting to recall that cows of reindeer and caribou normally possess antlers. The does of other species of deer have antlers only very exceptionally. Yet these spontaneously occurring instances of antler formation in females are said to be more common in *Capreolus* and *Odocoileus* than in other species of deer. The antlers carried by such individuals are described as being small and variously malformed. Furthermore, such atypical antlers are said invariably to retain their velvet and to be shed as a rule around the time of parturition. Atypical antlers permanently in velvet are reported as appearing occasionally on senile does (cf. Rörig, 1899, '07).

SUMMARY

An air gun has been devised with which slugs of testosterone propionate and other similar materials can be shot into deer, offering a convenient means of administering testosterone to them.

Castration of Virginia deer in the first eight months of life results in complete suppression of antler growth. Castration after the appearance of the first set of antlers, if the antlers are in the velvet, results in the permanent retention of the velvet and failure of the antlers to be shed. Castration, in the presence of antlers which have lost their velvet, results in immediate shedding of the antlers and their subsequent renewal and permanent retention in the following year.

Administration of testosterone to deer which have never had antlers as a result of castration as fawns induces antler growth.

Administration of testosterone to antlered deer, castrated as yearlings or thereafter, results in prompt shedding of the velvet and subsequent shedding of the antlers (tendency to restore normal antler cycle).

Administration of testosterone to normal male deer tends to induce shedding of the velvet; when the amount of hormone given is great, shedding occurs promptly. Moreover, by administering testosterone to normal males, shedding of the antlers can be postponed.

Administration of testosterone to ovariectomized female Virginia deer causes them to grow antlers.

Knowledge of the seasonal changes in antlers and gonads of deer,

combined with the observed effects of castration and administration of testosterone upon antler growth, leads us to postulate a regulation of the antler cycle by the testes and pituitary gland. The effects of castration of adult deer indicate the existence of a non-gonadal (hypophysial) factor responsible for antler growth and of a testicular factor responsible mainly for the secondary hardening (internal reorganization) of the antlers and loss of the velvet.

The failure of antlers to grow in deer castrated as fawns can be overcome by the administration of testosterone, indicating the necessity of an initial priming action of the testes; once so activated, however, the non-gonadal (hypophysial) stimulus for antler growth appears to become self-perpetuating and freed of the necessity of subsequent testicular priming. The annual onset of antler growth takes place at a time of minimal testicular function, shedding of the velvet upon increasing testicular activity, and antler shedding at a period of declining function of the testes. Thus, the anterior pituitary gland and gonads appear to alternate and supplement each other in regulating antler growth and maturation.

REFERENCES

BLAUEL, G.: *Endokrinologie* 15: 321. 1935.

BLAUEL, G.: *Endokrinologie* 17: 369. 1936.

CATON, J. D.: The Antelope and Deer of North America. New York. 1877.

NOBACK, C. V., AND W. MODELL: *Zoologica* 11: 19. 1930.

OLT, A.: *Brcht. Oberhess. Geschft. f. Natur- u. Heilk. zu Giessen* 11: 3. 1927.

OLT, A.: *Natur- u. Mus (Brcht. Senckenberg. naturforsch. Geschft.)* 58: 376. 1928.

RÖRIG, A.: *Arch. f. Entwcklngsmechn d. Organ.* 8: 382. 1899.

RÖRIG, A.: *Arch. f. Entwcklngsmeckn d. Organ.* 23: 1. 1907.

SCHUMACHER, S.: *Deutsch. Jäger* 45: 1. 1937.

TANDLER, J.: *Anz. Kais. Akad. d. Wissenschftn (Wien), math-natur. Kl.* 47: 252. 1910.

TANDLER, J., AND S. GROSZ: Die Biologischen Grundlagen der Sekundären Geschlechtscharaktere. Berlin. 1913.

TOWNSEND, M. T., AND M. W. SMITH: *Rooesvelt Wild Life Bull.* 6: 161. 1933.

WISLOCKI, G. B.: *Am. J. Anat.* 71: 371. 1942.

WISLOCKI, G. B.: Essays in Biology. Univ. Calif. Press. 631. 1943.

NOTES AND COMMENTS

THIOURACIL ACTION IN STARVED RATS

THE ACTIVITY of endocrine glands as well as the response of target organs to exogenous hormones is influenced by the nutritional condition of the body. Since in partial starvation there is on the one hand decreased pituitary function, on the other increased sensitivity of many target organs it appeared of interest to study the effect of thiouracil administration in partially starved rats.

Material and methods. Adult male rats were used. Animals were kept in individual wire screen cages. Control animals had free access to stockfood (purina dog chow). "Starved" rats received 1 pellet (8–9 gm.) daily. All animals had free access to fluid, tap water or thiouracil solution (0.05%) respectively. Weights were recorded daily. At the end of the experiments the animals were killed, the thyroid glands were fixed in Bouin's fluid, sectioned and stained with hematoxilin-eosin.

RESULTS

Experiment 1. Sixteen rats were starved for three weeks prior to thiouracil administration. During this time they lost 25–33% of their initial bodyweight. From the twenty-second day of starvation on their received 0.05% thiouracil solution instead of tap water, the starvation ration being continued. Sixteen normally fed controls received thiouracil solution for the same periods of time as the starved animals. Animals were killed in pairs on the third, fifth, seventh, and eleventh day of thiouracil treatment. In the normally fed controls there was histological evidence of stimulation of the thyroid glands as evidenced by increased cell height, vacuolization and loss of colloid, on the fifth day of thiouracil administration. Beginning changes were recognizable as early as two days. In contradistinction the starved rats showed no stimulation or only minimal traces up to six days of treatment. On the elventh day of thiouracil administration three out of the six starved animals showed a markedly stimulated thyroid, one showed a barely recognizable trace, and two had still failed to respond.

Experiment 2. In six rats thiouracil administration was started simultaneously with the starvation ration. Six normally fed controls received thiouracil at the same time. All animals were killed on the fifth day. The starved rats lost an average of 19% of bodyweight. The difference in the histological picture was not as marked as in the rats starved over longer periods, but even after this shorter starvation the response of the starved animals' thyroids was less than that of the controls.

DISCUSSION

Partial starvation is known to suppress secretion of several pituitary hormones, amongst them thyrotrophic hormone. The thyroid glands of such

Received for publication January 9, 1947.

animals have been found more sensitive to exogenous thyrotrophic hormone than normally fed controls, similar to the increased sensitivity of the thyroids of hypophysectomized animals (Stephens, 1940). Thiouracil normally induces release of increased amounts of thyrotrophic hormone by inhibiting formation of thyroid hormone, thereby taking the brakes off the pituitary gland (MacKenzie and MacKenzie, 1943, Astwood et al., 1943), and it may also directly enhance the action of thyrotrophic hormone thus liberated (Rawson et al., 1946).

The lessened response to thiouracil of the thyroids of partially starved rats as described in this report, could be due to the decreased function of the anterior pituitary. On the other hand in starvation thiouracil acts upon a thyroid gland which is already secreting subnormal amounts of thyroid hormone. The decrease of circulating thyroid hormone induced by thiouracil must then be of a lesser magnitude in the starved than in the normal animals. Consequently, there is a weaker stimulus for the pituitary to secrete thyrotrophic hormone. The starved animal resembles in this respect the animal kept at high environmental temperatures. Such animals also show a decreased response to thiouracil administration (Dempsey and Astwood, 1944; Paschkis et al., 1945).

The decreased response of the thyroid of starved animals to thiouracil is probably due to both the hypofunction of the anterior pituitary, and the weaker stimulus exerted upon the pituitary. The fact that the sensitivity of the thyroid gland to thyrotrophic hormone under conditions of starvation is increased (Stephens, 1940) indicates that the thyrotrophin secretion under the conditions of the experiment must be very low.

SUMMARY

In partially starved rats the hypertrophy and hyperplasia of the thyroid gland induced by thiouracil is less marked and develops later than in normally fed controls.

REFERENCES

Astwood, E. B., J. Sullivan, A. Bissel and R. Tyslowitz: *Endocrinology* 32: 210. 1943.

Dempsey, E. W., and E. B. Astwood: *Endocrinology* 32: 509. 1944.

MacKenzie, C. G., and J. B. MacKenzie: *Endocrinology* 32: 185. 1943.

Paschkis, K. E., A. Cantarow, A. E. Rakoff and M. S. Rothenberg: *Endocrinology* 37: 133. 1945.

Rawson, R. W., A. Albert, J. McArthur, P. Merrill, B. Lennon and Ch. Ridell: *Endocrinology* 39: 74. 1946.

Stephens, E.: *Endocrinology* 26: 485. 19490.

F. Gomez Mont,[1] K. E. Paschkis, and A. Cantarow

[1] Present address Calle del Cedro 48-A, Mexico, D. F.

THE REACTION OF THE MALE SOUTH AFRICAN CLAWED FROG (XENOPUS LAEVIS) TO GONADOTROPINS[1]

RESEARCH on the South African clawed frog (Xenopus laevis) has been limited largely to investigations on the female frog (Shapiro and Zwarenstein, 1934), (Bellerby, 1934). Its sensitivity to gonadotropic hormones has been extensively studied and the value of this amphibian in pregnancy diagnostic tests, as well as in endocrinologic bio-assays, is widely recognized.

At the present time, knowledge of the effect of gonadotropic hormones on the male is limited chiefly to the use of this hormone in the male frog in breeding experiments for the production of amplexus (copulatory embrace). This method of obtaining fertilized eggs, described by Shapiro in 1936, has made possible the breeding and rearing of these frogs in captivity. By inference, therefore, it was realized that the male frog was stimulated into the extrusion of spermatozoa by mammalian gonadotropic hormones. To the best of our knowledge, no actual demonstration of this reaction in the male to gonadotropins has been reported.

In an effort to investigate the rather numerous failures in breeding experiments with the South African clawed frog, inevitably encountered by all investigators in this field, cloacal smears of the male frog were performed by us at intervals after the administration of gonadotropic hormones. The simplicity with which this reaction could be followed, namely, the microscopic visualization of actively motile spermatozoa, as well as the speed with which the reaction developed following the administration of the hormone, was striking. Actively motile spermatozoa, not too dissimilar from human spermatozoa, were observed within 2 to 3 hours of the injection of hormone. Further study revealed that the male not only reacted more quickly than the female, but that it moreover was considerably more sensitive to gonadotropic hormones than the female frog, as well as the immature rat. It was possible to titrate the strength of hormone preparations by the administration of serial dilutions. The potential value and the possible use of this amphibian for bio-assay methods was at once obvious. The relative sensitivity of the male frog, the female frog and the immature rat to gonadotropic hormones of pituitary and chorionic origin was studied and is reported in the present communication.

After the administration of the hormone, all injections being given subcutaneously in the dorsal lymph sac of the frog, drops of cloacal fluid are removed at periodic intervals by the use of a blunt-tipped, glass capillary pipette. It is sufficient to obtain this cloacal fluid at the external orifice of the cloacal tract, since attempts to enter the cavity with the glass pipette are not only hazardous but are moreover unnecessary. Ample fluid can always be obtained at the outlet when the test is positive.

The inability to obtain an adequate amount of cloacal secretion is sufficient indication by itself that the reaction is negative and that further efforts are for the time being unnecessary.

The fluid thus obtained is placed directly on a coverslip and this is inverted on a ring of vaseline on a glass slide and examined with a microscope

Received for publication February 6, 1947.
[1] This investigation was aided in part by a grant from Ciba Pharmaceutical Products, Inc., Summit, New Jersey.

with the light reduced. Actively motile spermatozoa are readily seen. Definite positive reactions are characterized by numerous actively motile spermatozoa found in virtually every field examined. The presence of non-motile spermatozoa, or 1 to 4 motile spermatozoa per high power field, should not be considered a positive result since they may rarely be found, even in non-injected frogs.

In general, using gonadotropic hormones, positive reactions develop within 1½ to 2 hours after the administration of the hormone. The onset of the appearance of spermatozoa may be quite abrupt, and a completely negative preparation may be replaced by one full of actively motile spermatozoa within a half hour. However, in general, the reaction develops progressively, reaching a peak within 2 to 3 hours after administration of the hormone. In instances of "weak positives," the spermatozoa may begin to disappear within 3 to 4 hours after their initial appearance. However, even in the weakest responses to the hormones, spermatozoa can be observed for periods of at least 2 to 3 hours and hence periodic examination spaced 1 to 1½ hours apart can be relied upon to detect the presence of a positive reaction. Occasionally strongly positive tests will result in continuous extrusion of spermatozoa for 12 to 20 hours. If examinations are repeatedly negative for 4 to 5 hours after the hormone injection has been given, it may safely be assumed that the test is negative.

After the completion of a positive test, it has been our custom to rest the animal for a 7 day period, at which time it is again ready for use. Somewhat shorter rest periods of 3 to 4 days suffice following a negative test. Like the female, these male frogs may be used repeatedly.

By the use of the above-described technic, gonadotropic hormones of sheep pituitary origin[2] and chorionic gonadotropic hormones[3] were titrated in male and female frogs and in immature rats. The procedure followed consisted in administering graded doses of the hormones into a series of each type of animal to determine the smallest amount of hormone required to produce a positive reaction. All the animals were run in pairs. For the rat the reaction required was the formation of corpora lutea in the ovary.

Using these methods the following comparative results were obtained: The male frog was found to be 64 times as sensitive to pituitary gonadotropic hormones as the female, and 128 times as sensitive as the rat. Furthermore, the male proved to be twice as sensitive as the rat to chorionic gonadotropic hormone and 10 times as sensitive as the female frog.

These relationships can be summarized as follows: For pituitary gonadotropins 1 rat unit is equivalent to 2 female frog units and 128 male frog units; for chorionic gonadotropins 1 rat unit is equivalent to one-fifth of a female frog unit and 2 male frog units. It should be noted that the sensitivities of the male and female frogs compared to each other and to the rat are distinctly different for gonadotropins of pituitary and chorionic origin.

In conclusion, therefore, it can be said that a new laboratory animal, the *male* South African clawed frog, is now available for endocrinologic assay, particularly in the field of sex hormones. This animal is many times more

[2] "Gonadophysin," supplied in part through the kindness of G. D. Searle & Co., Chicago.

[3] Commercial preparation distributed by the Physicians Drug & Supply Co., Philadelphia.

sensitive to pituitary and chorionic gonadotropins than the standard laboratory animals now used for these assays, thus permitting the detection of levels of hormone too small to be determined by methods heretofore available. Moreover, it possesses the significant advantage of enabling an assay to be carried out within a few hours instead of requiring, as it does with other animals, one to several days. The obvious applicability of this new test to the field of pregnancy diagnosis and other endocrinological studies is now under investigation.

SUMMARY

The male South African clawed frog (Xenopus laevis) was found to be a satisfactory animal for assays of gonadotropins of both pituitary and chorionic origins. It is more sensitive and reacts far more quickly than any animal heretofore employed for the detection of such hormones.

STANLEY L. ROBBINS, FREDERIC PARKER, JR., AND PHYLLIS D. BIANCO
From the Mallory Institute of Pathology, Boston City Hospital

REFERENCES

BELLERBY, C. W.: *Nature, London* 133: 494. 1934.
SHAPIRO, H. A.: *J. Exper. Biol.* 13: 48. 1936.
SHAPIRO, H. A., AND H. ZWARENSTEIN: *Nature, London* 133: 339. 1934.
SHAPIRO, H. A., AND H. ZWARENSTEIN: *Nature, London* 133: 762. 1934.

NEW BOOK

VITAMINS AND HORMONES, volume IV, 1947. Edited by R. S. Harris and K. W. Thimann. Published by the Academic Press, Inc. 125 E. 23rd St., New York City. xvii+406 pp. $6.80.

Four years ago, the Editors of *Vitamins and Hormones* launched an ambitious undertaking. They proposed to publish an annual volume of reviews dedicated to the proposition that vitamins and hormones are interrelated. To the casual reader there seemed then to be little systematic data to support this contention. Endocrinologists had learned that animals in good nutritional states were required for eliciting many hormonal effects, but, as Hertz remarks in the current volume, the effects of dietary restriction were commonly regarded as non-specific and therefore of little significance. Nutritionists, in describing deficiency effects, were aware of disturbances in endocrine systems and of lesions in endocrine organs, but there were few attempts to explain the symptoms of deficiencies by considering the changed endocrine status. Each specialty went its own way without regard to the doings of the other.

That these attitudes have changed rapidly is now apparent to any one who reads endocrine literature. A number of specific deficiencies are known to cause profound disturbances in adrenal cortical function. These disturbances sometimes produce overactivity of the gland so severe as to cause its ultimate exhaustion. Such overproduction of cortical hormones may cause far-reaching effects, on protein and carbohydrate metabolism, on lymphocyte destruction and antibody formation and on pathological changes in such

widely disparate structures as blood vessels and joints. Other examples are easily brought to mind. Two chapters in the current volume consider the effects of B vitamins on reproduction and the nutritional therapy of endocrine disturbances. The discovery that thiouracil and thiourea, substances related to the B vitamins in that they may be considered as prosthetic parts of nucleoproteins and nucleotides, prevent the formation of thyroid hormone suggests the need for determining the amount of antithyroid agents in foodstuffs. Tracing out the mechanisms involved in such nutritional situations and evaluating the part the deficiencies play in disease is a fascinating prospect for the endocrine physiologist.

Enough has been said to indicate how sound was the judgment of the Editors when the series was begun. It is, of course, impossible to identify accurately the forces which caused the increased interest in nutritional and endocrine interrelationships, but the least that can be said is that *Vitamins and Hormones* has called the turn. It may well be that these excellent reviews have forcibly determined the present trends.

In the earlier volumes, the material was divided between nutritional chapters and chapters dealing for the most part with purely endocrine effects. The fourth and current volume achieves a much better synthesis, although perfect accord between the representatives of the two fields has not yet been reached. Nevertheless, much ground has been gained, and this reviewer can only reiterate his opinion, expressed on the publication of the first volume, that the breadth and amount of material encompassed, and above all, the emphasis on further and more critical investigation of the tentative conclusions, make it necessary to add this series to the reference books available to all investigators in the fields of nutrition and endocrinology.

EDWARD W. DEMPSEY